Morphokinetic assessment, enabled by time lapse imaging *in vitro*, to enhance knowledge of preimplantation human embryo development and improve clinical outcomes

A thesis submitted to the University of Kent for the degree of

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Declaration

No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Kent or any other University or institute of learning.

Alison Campbell

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Incorporation of published and presented work

This thesis encapsulates individual published manuscripts, abstracts, book chapters and oral presentations, as follows:

Published manuscripts 2011 to date

Simon Fishel and **Alison Campbell**. Embryo Selection for IVF – is there a signal in the noise? Focus on Reproduction, cover story. September 2011.

A Campbell, S Fishel, N Bowman, S Duffy, M Sedler, C Fontes Lindemann Hickmann
Modelling a risk classification of aneuploidy in human embryos using non-invasive morphokinetics
Reproductive BioMedicine Online (2013) 26, 477-485
(Secured a runner up position in the 2013 'Robert G. Edwards Prize Paper Award')

A Campbell, S Fishel, N Bowman, S Duffy, M Sedler, S Thornton
Retrospective analysis of outcomes after IVF using an aneuploidy risk model derived from time-lapse imaging without PGS
Reproductive BioMedicine Online (2013) 27, 140-146
(Certificate received for 'Highly Cited research' Nov 2016)

Alison Campbell, Simon Fishel, Mette Laedgdsmand
Aneuploidy is a key causal factor of delays in blastulation detected by time lapse imaging. Author response to 'A cautionary note against aneuploidy risk assessment using time lapse imaging'.
Reproductive Biomedicine Online (2014) 28, 279-283

Kirkegaard K, **Campbell A**, Agerholm I, Bentin-Ley U, Gabrielsen A, Kirk J, Sayed S, Ingerslev HJ. Limitations of a time-lapse blastocyst prediction model: a large multicentre outcome analysis. Reproductive biomedicine online. 2014 Aug 1;29(2):156-8.

Ciray HN*, **Campbell A***, Agerholm IE, Aguilar J, Chamayou S, Esbert M, Sayed S. Proposed guidelines on the nomenclature and annotation of dynamic human embryo monitoring by a time-lapse user group. Human Reproduction. 2014 Oct 24;29(12):2650-60.
(Joint first author)

MN Shahbazi, A Jedrukik, S Vuoristo, G Recher, A Hupalowska, V Bolton, NNM Fogarty, **A Campbell**, LD Gasparini, D Ilic, Y Khalaf, KK Niakan, S Fishel, M Zernicka-Goetz, Self-organisation of the human embryo in the absence of maternal tissues.
Nature Cell Biology, 18, 700-708, 2016

S Fishel, **A Campbell**, S Montgomery, R Smith, L Nice, S Duffy, L Jenner, K Berrisford, L Kellam, R Smith, I D'Cruz, A Beccles, Live births after embryo selection using morphokinetics versus conventional morphology: a retrospective analysis.
Reproductive BioMedicine Online, 2017 Oct; 35(4): 407-416

ESHRE Special Interest Group of Embryology, Alpha Scientists in Reproductive Medicine. The Vienna consensus: report of an expert meeting on the development of art laboratory performance indicators. 2017

Kellam L, Pastorelli LM, Bastida AM, Senkbeil A, Montgomery S, Fishel S, Campbell A, Perivitelline threads in cleavage-stage human embryos: observations using time-lapse imaging. Reproductive BioMedicine Online. 2017 Dec;35(6):646-656. doi: 10.1016/j.rbmo.2017.09.004. Epub 2017 Sep 28

S Fishel, **A Campbell**, S Montgomery, R Smith, L Nice, S Duffy, L Jenner, K Berrisford, L Kellam, R Smith, F Foad, Ashley Beccles. Time-lapse imaging algorithms rank human preimplantation embryos according to the probability of live birth. Reproductive BioMedicine Online, 2018; 37 (3): 304-313

Simon Fishel, **Alison Campbell** et al. Evolution of embryo selection for IVF from subjective morphology assessment to objective time-lapse algorithms improves chance of live birth. Reproductive BioMedicine Online *in press October 2019*

There is only one thing that is truly important in an IVF lab: Everything. Cairo Consensus Guidelines on IVF Culture Conditions.
Reproductive BioMedicine Online In press *Dec 2019*

Books and chapters

Alison Campbell, Simon B Fishel (eds). Atlas of Time Lapse Embryology. CRC Press, 2014

Alison Campbell. Time-lapse, the cell cycle, distribution of morphokinetic timings and known implantation data. Chapter 3, in Atlas of Time Lapse Embryology, CRC Press 2014

Alison Campbell. Clinical aspects of time lapse embryology. Chapter 2, in Atlas of Time Lapse Embryology, CRC Press 2014

Alison Campbell. Noninvasive techniques: Embryo selection by Time-lapse imaging. A practical guide to selecting gametes and embryos. Markus Montag (ed.). CRC Press, 2013

Ahlström A, **Campbell A**, Ingerslev HJ, Kirkegaard K. Prediction of Embryo Viability by Morphokinetic Evaluation to Facilitate Single Transfer. In Screening the Single Euploid Embryo 2015 (pp. 133-145). Springer, Cham.

Alison Campbell, Patricia Collins. Preimplantation development. Section 2 : Embryogenesis. Standring S, editor. Gray's Anatomy: The anatomical basis of clinical practice. Elsevier Health Sciences; 2015

Abstracts (2012 – 2020)

2012

A Campbell, S Duffy, L Best, K R Jordan, N Bowman, M Sedler, S Fishel
Assessment of early embryo development by the EmbryoScope™ (Unisense, Denmark) in relation to oocyte ploidy: ACE 8th Annual Conference, Leeds, January 2012

C Drew, C Hickman, L Best, N Bowman, K Jordan, S Duffy, A Campbell, S Fishel
A time lapse investigation using the EmbryoScope™ (Unisense, Denmark) into the timing of PB2 extrusion to review the standard operating procedure for clinical preimplantation genetic screening (PGS) ACE 8th Annual Conference, Leeds, January 2012

N Bowman, L Best, A Campbell, K Jordan, K Gardner, M Sedler, S Fishel
The first UK cases of trophectoderm biopsy for aneuploidy screening of blastocysts, using Gene Security Network's SNP array and 'Parental Support': ACE 8th Annual Conference, Leeds, January 2012

N Bowman, A Campbell, L Best, K Jordan, K Gardner, M Sedler, C Lynch, S Fishel
Inference of oocyte ploidy following 1st (PB1) and 2nd (PB2) array CGH. Initial experiences and clinical results: ACE 8th Annual Conference, Leeds, January 2012

L Best, A Campbell, L Lee, N Bowman, E Power, KR Jordan, K Harrison, S Duffy, L Kellam, S Fishel
'Rescue' ICSI of late matured oocytes following failed intracytoplasmic sperm injection (ICSI) fertilisation: ACE 8th Annual Conference, Leeds, January 2012

A Burchill, A Campbell, S Fishel, R Smith, D Hulme, C Drezet, S Foley, C Shearer, C Barton
A retrospective audit of fast-cleaving fresh embryos in 83 extended culture (EC) cycles: ACE 8th Annual Conference, Leeds, January 2012

Duffy, A Campbell, L Best, S Fishel
The UK's first EmbryoScope™ IVF treatment cycles: an assessment of 92 patient outcomes: ACE 8th Annual Conference, Leeds, January 2012

R Smith, A Burchill, C Drezet, S Foley, C Shearer, A Campbell, S Fishel
Elective single blastocyst transfer, with suitable supernumerary embryos vitrified versus day 3 (D3) elective single embryo transfer (eSET) and day 3 double embryo transfer (DET) with conventionally frozen supernumerary embryos. A comparison of cumulative clinical pregnancy rates (CPR). Which is the better policy? : ACE 8th Annual Conference, Leeds, January 2012

S Fishel, E Cater, C Lynch, L Jenner, A Campbell
Polar body 1, polar body 2 and blastomere analysis in PGS treatment: PGDIS, Bregenz, Austria, May 2012

A Campbell, CFL Hickman, S Duffy, N Bowman, K Gardner, S Fishel
Morphokinetics of 'Aneuploid' and 'Euploid' human embryos, inferred by polar body or trophectoderm biopsy: ESHRE 28th Annual Meeting, Istanbul, July 2012

CFL Hickman, A Campbell, S Duffy, S Fishel
'Reverse Cleavage': its significant with regards to human embryo morphokinetics, poloidy and stimulation protocol: ESHRE 28th Annual Meeting, Istanbul, July 2012

E Cater, C Lynch, L Jenner, K Berrisford, A Campbell, N Keown, H Rouse, A Craig, S Fishel
Mosaicism with the day 3 embryo previously inferred as aneuploidy from the first polar body: ESHRE 28th Annual Meeting, Istanbul, July 2012

L Best, A Campbell, S Fishel
Multicentre Assessment of survival and viability of vitrified blastocysts stored in nitrogen vapour compared with those stored in liquid nitrogen: ESHRE 28th Annual Meeting, Istanbul, July 2012

K Gardner, A Campbell, C Clayton, G Atkinson, S Fishel
An investigation into two clusters of monozygotic twinning following assisted conception in a large UK IVF facility. Can we identify patients at higher risk? ESHRE 28th Annual Meeting, Istanbul, July 2012

N Bowman, A Campbell, S Duffy, K Gardner, M Sedler, S Fishel
Changing Culture Conditions. Blastocyst ploidy following standard incubation and uninterrupted culture in the Embryoscope: American Society for Reproductive Medicine 68th Annual Meeting, San Diego, California, October 2012

2013

C Shearer, A Burchill, C Drezet, S Foley, R Smith, A Shaker, S Fishel, A Campbell
A case study reporting Schistosoma haematobium miracidia detected in semen and the outcome on treatment decision: Fertility 2013, Liverpool, UK, January 2013

L Best, S Montgomery, A Campbell, S Fishel
Standard Incubation versus Embryoscope Incubation: a retrospective analysis of pregnancy outcome: Fertility 2013, Liverpool, UK, January 2013

K Gardner, S Montgomery, A Campbell, S Fishel
An investigation into the effect of storing sperm in the dark between the time of preparation and insemination: Fertility 2013, Liverpool, UK, January 2013

S Montgomery, S Duffy, N Bowman, M Sedler, A Campbell, S Fishel
Does the timing and degree of cleavage stage embryo fragmentation differ in Euploid and Aneuploid Embryos? Fertility 2013, Liverpool, UK, January 2013

A Campbell, C Fontes Lindemann Hickman, S Duffy, S Fishel
Comparison of morphokinetic variables of implanted and non-implanted embryos. A search for exclusion criteria: Fertility 2013, Liverpool, UK, January 2013

A Campbell, C Fontes Lindemann Hickman, S Duffy, N Bowman, M Sedler, S Fishel
A non-invasive time-lapse model to classify the risk of aneuploidy in embryos: Fertility 2013, Liverpool, UK, January 2013

MJ Sedler, N Bowman, A Campbell, K Jordan, L Best, D Moult, S Fishel
PGS using SNP array and Parental Support technology to evaluate embryo ploidy status via trophectoderm biopsy: Clinical outcome: Fertility 2013, Liverpool, UK, January 2013

L Best, A Campbell, S Duffy, S Montgomery, S Fishel
Does one model fit all? Testing a published embryo selection algorithm on independent time-lapse data: ESHRE Annual Meeting, London, UK, July 2013

E Cater, D Hulme, L Jenner, A Campbell, S Fishel
Retrospective analysis of clinical outcome of vitrified blastocyst post thaw using time-lapse imaging and standard incubation: ESHRE Annual Meeting, London, UK, July 2013

N Bowman, S Montgomery, L Best, A Campbell, S Duffy, S Fishel
Comparison of clinical results between standard and uninterrupted embryo culture, where there are no embryos to select between: ESHRE Annual Meeting, London, UK, July 2013

S Duffy, A Campbell, S Montgomery, C Hickman, S Fishel
Does gender affect pre-implantation embryonic development rates? Looking back at the baby pictures: ESHRE Annual Meeting, London, UK, July 2013

E Power, S Montgomery, S Duffy, K Jordan, A Campbell, S Fishel
Can completion of compaction predict implantation outcome?: ESHRE Annual Meeting, London, UK, July 2013

A Campbell, S Fishel, S Duffy, S Montgomery
Embryo selection model defined using morphokinetic data from human embryos to predict implantation and live birth: Conjoint Meeting of the International Federation of Fertility Societies and the American Society for Reproductive Medicine, Boston, Massachusetts, October 2013

A Campbell, S Fishel, E Cater et al
Retrospective assessment of time lapse data from 6730 human embryos to establish incidence rates for preimplantation cleavage anomalies: ACE, Sheffield, UK, January 2014
Oral presentation – best presentation prize

A Campbell, S Fishel, S Montgomery, L Jenner, L Nice, R Smith, S Duffy, D Hulme, D Sivanantham
Inter clinic comparison of morphokinetic variables: ACE, Sheffield, UK, January 2014

CF Drew, S Montgomery, A Campbell, S Fishel
Prospective randomised controlled trial to assess the effect of EmbryoGlue as a medium for embryo transfer: ACE, Sheffield, UK, January 2014

D Hulme, L Jenner, A Campbell, S Fishel
A randomised controlled comparison of clinical outcome, following time lapse and standard incubation: ACE, Sheffield, UK, January 2014

S Sivanantham, LR Nice, A Campbell, S Fishel
A comparative study of pronuclear fading times and implantation using embryos cultured using time lapse imaging: ACE, Sheffield, UK, January 2014

S Sivanantham, LR Nice, A Campbell, S Fishel
Validation of pronuclear assessment timings using embryos cultured using time lapse imaging: ACE, Sheffield, UK, January 2014

E Cater, A Campbell, L Jenner, S Fishel
Preliminary retrospective study investigating the incidence and significance of cytoplasmic strings in the human blastocyst: ACE, Sheffield, UK, January 2014

F Toal, G Emerson, A Campbell, S Fishel
Introduction of a PGD program into the Republic of Ireland under the EUTCD: ACE, Sheffield, UK, January 2014

K Gardner, S Montgomery, L Best, A Campbell, S Fishel
Case Report: A successful fertilisation and ongoing pregnancy following calcium ionophore artificial oocyte activation after recurrent failed fertilisation with ICSI: ACE, Sheffield, UK, January 2014

L Best, A Campbell, S Montgomery, R Smith, S Duffy, D Hulme, D Savanantham, L Jenner, L Nice, S Fishel
Does female age matter when selecting blastocysts for transfer using a novel morphokinetic based blastocyst selection algorithm?: ESHRE 2014, Munich, Germany, June/July 2014

R Smith, A Campbell, M Laegdsmand, S Montgomery, L Best, S Duffy, D Hulme, D Sivanantham, L Jenner, L Nice, S Fishel
The use of live birth as an outcome measure for a new morphokinetic based blastocyst selection algorithm: ESHRE 2014, Munich, Germany, June/July 2014

M Regueira, E Cater, L Jenner, G Woodhead, C Lynch, A Campbell, S Fishel
Retrospective assessment of second polar body alignment using time lapse imaging: ESHRE 2014, Munich, Germany, June/July 2014

L Lee, S Montgomery, S Duffy, K Jordan, A Campbell, S Fishel
Is it necessary to refresh Global medium on D3 during EmbryoScope culture?: ESHRE 2014, Munich, Germany, 2014

S Montgomery, A Campbell, M Laegdsmand, R Smith, L Best, D Hulme, D Sivanantham, L Jenner, L Nice, S Fishel
The relationship between early cleavage morphokinetic time points is more predictive of implantation and live birth than single time points alone: ESHRE 2014, Munich, Germany, June/July 2014

2015

S Rhodes, L Nice, A Campbell, S Fishel
Morphokinetic comparison of culture medium: LifeGlobal® supplemented with 10% Dextran Serum supplementation vs Irvine Scientific Continuous Single Culture™: Fertility 2015, Birmingham, January 2015

C Drezet, R Smith, A Burchill, S Foley, C Shearer, A Campbell, S Fishel
Are split IVF-ICSI cycles an effective way to managing unexplained infertility?: Fertility 2015, Birmingham, January 2015

C Shearer, R Smith, A Campbell, S Fishel
The time interval between 1 and 7 hours from oocyte recovery to IVF insemination has no impact on outcome data: Fertility 2015, Birmingham, January 2015

S Foley, R Smith, C Drezet, A Campbell, S Fishel
To re-freeze or not to re-freeze? That is the question: Fertility 2015, Birmingham, January 2015

A Campbell, L Best, S Duffy, S Montgomery, R Smith, D Hulme, D Sivanantham, K Berrisford, N Bowman, R Smith, L Jenner, S Fishel
Time lapse study of the duration, distribution and incidence of specific irregular cleavage patterns in 8988 early embryos: Fertility 2015, Birmingham, January 2015

A Campbell, S Fishel, D Griffin
Ploidy, morphokinetics and time lapse imaging: The story so far: Fertility 2015, Birmingham,

D Hulme, L Jenner, A Campbell, S Fishel
The incidence of reverse cleavage events identified by time lapse imaging of 13823 embryos: Fertility 2015, Birmingham, January 2015

E Power, S Montgomery, N Bowman, K Gardiner, A Campbell, S Fishel
A study of blastomere arrangement at the 4 cell stage in transferred embryos with known clinical outcome: Fertility 2015, Birmingham, January 2015

K Gardner, R Smith, N Bowman, E Power, S Duffy, S Fishel, A Campbell
A study of vacuolation in transferred human preimplantation embryos which resulted in live birth, compared with those which did not: Fertility 2015, Birmingham, January 2015

K Berrisford, A Campbell, E Cater, L Jenner, S Fishel
Retrospective study of patients using EmbryoGen for embryo culture based on previous clinical history: Fertility 2015, Birmingham, January 2015

L Jenner, A Campbell, R Smith, S Montgomery, S Fishel
Introduction of D4 embryo transfer in an IVF clinic: Fertility 2015, Birmingham, January 2015

N Bowman, K Gardner, E Power, S Montgomery, S Duffy, S Fishel, A Campbell
Negative association between multichotomous mitosis of blastomeres within cleavage stage embryos and live birth outcomes in 347 embryos: Fertility 2015, Birmingham, January 2015

A Burchill, R Smith, C Drezet, A Campbell, S Fishel
Case report: the possible causes and effects of non-apposition of pronuclei in three out of seven embryos cultured in time-lapse culture: Fertility 2015, Birmingham, January 2015

A Campbell; M Laegdsmand; S Fishel; R Smith; S Montgomery; K Berrisford; R Smith; L Nice and DK Griffin
Large scale comparison of morphokinetic timings of over 12,500 IVF and ICSI embryos from insemination to blastulation: ESHRE 2015, Lisbon, Portugal, June 2015

C Lynch, D Maruthini, M Ragunath, L Jenner, S Fishel
First genetically confirmed monozygotic dichorionic diamniotic twin livebirth from a day 5 single blastocyst transfer: ESHRE 2015, Lisbon, Portugal, June 2015

Best L, Campbell A, Montgomery S, Smith R3, Duffy S, Hulme D, Sivanantham D, Jenner L, Nice L, Berrisford K and Fishel S
Is a morphokinetic based selection model for fresh blastocysts transferrable to vitrified blastocysts? : ESHRE 2015, Lisbon, Portugal, June 2015

Cater E, Jenner L, Campbell A, Fishel S
Can oocyte recipient outcome be predicted by oocyte donor age? : ESHRE 2015, Lisbon, Portugal, June 2015

2016

Berrisford K, Jenner, L, Campbell, A, Fishel, S
Polar body Vs. blastomere Vs. trophectoderm biopsy in CARE Nottingham PGS program (2008 onwards): ACE 2016 10th Biennial Conference, January 2016, Newcastle, UK

Montgomery, S., Campbell, A, Best, L, Davies, N, Power, E., Gardner, K, Fishel, S.
Are time-lapse morphokinetic algorithms corrected for patient age, really necessary: ACE 2016 10th Biennial Conference, January 2016, Newcastle, UK

Campbell A, Fishel, S, Theodorou, E, Montgomery, S, Smith, Rachel, Griffin, D, Laegdsmand, M
Morphokinetic comparison of 1620 human embryos resulting in, or failing to reach, live birth: ACE 2016 10th Biennial Conference, January 2016, Newcastle, UK

Cater, E, Campbell, A, Jenner, L, Fishel, S
Utilisation of embryos generated from ART: ACE 2016 10th Biennial Conference, January 2016, Newcastle, UK

Jordan K, Montgomery, S, Best, L, Campbell, A, Fishel, S
Sibling outcome study to compare outcome data between ICSI and IMSI: ACE 2016 10th Biennial Conference, January 2016, Newcastle, UK

Murphy L, Jenner L, Campbell A, Fishel S
Outcomes following multiple pregnancy in ART cycles: ACE 2016 10th Biennial Conference, January 2016, Newcastle, UK

Watchter A, Campbell, A, Fishel, S, Ventris, R, Montgomery, S, Nice, L, Jenner, L, Smith, Rachel, Paterson, Y.
Patient feedback on time lapse, results from an online survey of 1066 patients during a 33 months period: ACE 2016 10th Biennial Conference, January 2016, Newcastle, UK

L Jenner, E Cater, A Campbell, S Fishel
Analysis of PGS results based on day of biopsy and stage of blastulation: PGDIS, 15th International Conference on Preimplantation Genetic Diagnosis, May 2016, Bologna, Italy

A Campbell, R Smith, S Montgomery, L Jenner, L Nice, S Rhodes, K Dowell, S Fishel
A prospective multicentre comparison of two different single step culture media using sibling embryos: ESHRE 2016, 32nd Annual Meeting, July 2016, Helsinki, Finland

2017

C Lynch, L Jenner, A Campbell, T Gordon, D Griffin

Live birth following two rounds of trophectoderm biopsy, vitrification, and warming: Assessment of the efficacy of retesting PGD and PGS embryos: Fertility 2017, Newcastle.

Louise Kellam, Laura Pastorelli , Alison Campbell
Observation of commonly occurring Zona-Cytoplasmic Threads (ZCTs) in early embryo development: Fertility 2017, Newcastle.

George Woodhead, Ellen Armstrong, Louise Kellam, Lucy Jenner, Alison Campbell
Case Study: Double Blastocyst Transfer Leading to a Quadruplet Pregnancy: Fertility 2017, Newcastle.

K. Berrisford, L. Jenner, A. Campbell.
Retrospective study of patients using EmbryoGen®/BlastGenTM for embryo culture based on previous clinical history: Fertility 2017, Newcastle.

Samantha Dale, Laina Murphy, Ellen Armstrong , Lucy Jenner, Alison Campbell
A Follow-Up Study Comparing Implantation Rate to Blastocyst Grade in ART Cycles From January 2010 – December 2015: Fertility 2017, Newcastle.

Allison Benn, Ellen Armstrong, Lucy Jenner, Alison Campbell
Use of Thermocoins in laboratory quality control temperature monitoring: Fertility 2017

A Martin, L Kellam, J Graves, A Campbell, S Fishel
Cleavage Orientation in the Early Human Embryo – Considering the Perivitelline Space and the Zone Pellucida: IX Congreso ASEBIR, November 2017, Madrid, Spain

2018

A Campbell, I Gallos, M Regueira, S Montgomery, L Jenner, A Watcher, S Wheat, F Foad, R Smith, L Nice, S Fishel
Side by side: Angle of extrusion of second polar body, relative to position of the first polar body, is significantly associated with clinical outcome: Fertility 2018, Liverpool, UK

Sue Montgomery, Alison Campbell*. Embryo grading at compaction is highly predictive of outcome.* Fertility 2018, Birmingham.

Best, Louise; Montgomery, Sue; Nice, Lynne; Smith, Rachel; Jenner, Lucy; Campbell, Alison Campbell
A prospective multicentre comparison of sibling embryo morphokinetics using two different culture media: Fertility 2018, Birmingham.

M. Regueira, L. Best, C. Drew, K. Jordan, L. Lee, E. Power, M. Sedler, S. Montgomery, A. Campbell. *Effective use of preimplantation genetic screening following thawing, biopsy and re-vitrification of previously untested cryopreserved embryos*: Fertility 2018, Birmingham.

Drew C, Regueira M, Best L, Jordan K, Lee L, Power E, Sedler M., Montgomery S, Campbell A.
Re-Biopsy of 'No result' embryos: A valuable diagnosis? Fertility 2018, Birmingham.

Sam Rhodes, Judith Byrne, Lynne Nice, Mohammed Khairy, Rahnuma Kazem and Alison Campbell.
A 5 year review of live birth and multiple birth rate: Fertility 2018, Birmingham.

Angel M. Bastida, Louise Kellam, Joanne Graves, Lucy Jenner, Alison Campbell.
First cleavage orientation of the human embryo may be associated to a previous 'contact' with the zona pellucida (ZP): Fertility 2018, Birmingham.

Kathryn Minshall, Audrey Wachter, Mercedes Regueira, Sue Montgomery, Alison Campbell. *A study of the morphokinetics of blastocysts affected by aneuploidies compatible with life:* Fertility 2018, Birmingham.

M. Regueira, L. Best, C. Drew, K. Jordan, L. Lee, E. Power, M. Sedler, S. Montgomery, A. Campbell. *Preimplantation genetic screening on previously untested frozen embryos*: Fertility 2018, Birmingham.
Sam Rhodes, Mohammed Khairy, Lynne Nice, Rahnuma Kazem and Alison Campbell
Comparative Review of the use of artificial oocyte activation (AOA) in fresh ICSI cycles: Fertility 2018, Birmingham.

Joanne Graves, Andrea Blair, Lucy Jenner, Alison Campbell. *Our experience of new andrology inter-laboratory controls as part of the quality management system:* Fertility 2018. Birmingham.

M Regueira, A Campbell, I Gallos, S Montgomery, L Jenner, A Wachter, S Wheat, F Foad, R Smith, L Nice, E Arm
Fishel
Angle between first and second polar body is significantly associated with clinical outcome: Alpha 2018, Reykjavik, Iceland

Louise best, Alison Campbell . *Advanced blastocyst development stage, at the time of vitrification, is associated with increased implantation, regardless of morphology:* Alpha 2018, Reykjavik, Iceland

Alison Campbell, Ioannis Gallos, Fatima Mirzazadeh, Best Louise, Rachel Smith, Samantha Duffy, Fiona Foad, Sarah Thirlby, Stacy Wheat, Sue Montgomery, Sarah O'boyle, Laina Davies. *A time-lapse study of pronuclear fading in 38.944 embryos, following IVF or ICSI. Is it time to fine-tune practice for standard assessment?* Alpha 2018, Reykjavik, Iceland.

2019

A Campbell, L Best, R Smith, K Berrisford, S Montgomery, A Wachter, S Wheat, F Foad, L Nice, E Armstrong, S Fishel. *Time lapse analysis of 1185 human blastocysts resulting in live birth to assess whether gender affects the timing of preimplantation embryo development*: Fertility 2019, January 2019, Birmingham, UK

George Woodhead, Louise Kellam, Alison Campbell, Maha Ragunath, Kanna Jayaprakasan. *Odds and predictors of Monozygotic Twinning in a multi-centre cohort of 25,794 IVF cycles:* ESHRE 2018

Lucy Jenner, Alison Campbell, Louise Kellam, Kathryn Berrisford, *Four years of day 4 embryo transfer:* Fertility 2019,

Caroline Rossiter, Ellen Armstrong, Alison Campbell. *Managing expectations of patients with a single oocyte collected – a retrospective analysis of outcomes:* Fertility 2019, Birmingham.

Sofia Tsagdi, Ellen Armstrong, Sue Montgomery and Alison Campbell. *Does intracytoplasmic sperm injection improve fertilisation rate and pregnancy outcome, compared with IVF, in patients with non-male factor infertility when 3 or fewer oocytes are retrieved?* Fertility 2019, Birmingham.

Alison Campbell; Louise best; Rachel Smith; Kathryn Berresford; Sue Montgomery; Audrey Wachter; Stacey Wheat; Fiona Foad; Lynne Nice ;Ellen Armstrong; Simon Fishel. *Time lapse analysis of 1185 human blastocysts resulting in live birth to assess whether gender affects the timing of preimplantation embryo development:* Fertility 2019, Birmingham.

2020

Margarida Avo Santos, Iria Castro, Alexandra Page, Amanda Tozer, Sue Montgomery, Alison Campbell
Can artificial oocyte activation using calcium ionophore improve blastulation rates in women with previous failed or low blastulation yield? Fertility 2020, Edinburgh.

Campbell, Alison; Smith, Rachel; Best, Louise; Montgomery, Sue; Berrisford, Kathryn; Nice, Lynne; Armstrong, Ellen; Lodge, Yvonne; Page, Alex;
Corcoran, Sharon; Drezet, Cath. *'Group culture': Embryology perspectives of working within a large cohesive group of IVF* clinics: Fertility 2020, Edinburgh.

Carmen Miralles Cuadrado, Fatima Mirzazadeh, Mohammed Khairy, Ellen Armstrong, Megan Lockwood, Lynne Nice, Louise Best, Alison Campbell. *Can blastocyst percentage re-expansion after warming be used to predict implantation potential and live birth outcome?* Fertility 2020, Edinburgh.

Rachel Smith, Alison Campbell. *Types of vacuolation, in the human preimplantation embryo and association with live birth:* Fertility 2020, Edinburgh.

Natalie Davis, Alison Campbell. *Cell fusion in the preimplantation human embryo is not associated with a significantly decreased live birth rate:* Fertility 2020, Edinburgh.

Ellen Armstrong, Alison Campbell. *Do we need a laser to perform biopsy for PGT-A?* Fertility 2020, Edinburgh.

Sue Montgomery, Alison Campbell. *Classification of embryos at the morula stage is highly correlated with live birth outcome:* Fertility 2020, Edinburgh.

Sarah Thirlby-Moore, Ellen Armstrong, Alison Campbell. *Which donor sperm bank will give me the best chance of success? – A multicentre, retrospective study:* Fertility 2020, Edinburgh.

Carmen Miralles, Alison Campbell. *Can blastocyst percentage of re-expansion after warming be used to predict implantation potential and live birth outcome?* Fertility 2020, Edinburgh.

Margarida Avo Santos, Iria Castro, Alexandra Page, Sue Montgomery, Alison Campbell. *Can artificial oocyte activation using calcium ionophore improve blastulation rates in women with previous failed or low blastulation yield?* Fertility 2020, Edinbugh.

Thesis-related presentations by invitation (2011-2019), Alison Campbell

- Billund, Denmark. Autumn 2011 EmbryoScope workshop

First UK clinical results following EmbryoScope culture and a preliminary look at ploidy.

- Billund, Denmark. Autumn 2012 EmbryoScope workshop

Retrospective data analysis – looking into ploidy and prediction

- Billund, Denmark. Spring 2014
    1. Use of the EmbryoScope™ time-lapse system to determine the real incidence of transient or aberrant phenomena in the human preimplantation embryo
    2. The EmbryoScope™ time-lapse system at CARE Fertility - impact on staff and patients

- London. Precongress lecture: ALPHA 2012

Time lapse and morphokinetics

- Istanbul. Precongress lecture: ESHRE 2012

Time lapse in the IVF laboratory.

- Precongress German embryology society 2012

Adapting to life with the EmbryoScope, experiences and commercial implementation

- Istabnul. Precongress PGDIS 2013

Use of the EmbryoScope in modeling a risk classification for aneuploidy in human embryos.

- Leipzig- German society of embryologists April 2013

Use of the EmbryoScope in modeling a risk classification for aneuploidy in human embryos.

- Innsbruck - Austrian society of embryologists September 2013

Aneuploidy risk classification model using the EmbryoScope.

- Paris - IVF DIAC 2014, 18 Jan 2014

Time lapse and embryo aneuploidy

- Rome – SIERR (Italian society of reproduction and embryology) annual conference. 7 Feb 2014

Are morphokinetics and other biomarkers of embryo quality clinically applicable?

- Canterbury PGDIS 2014

Morphokinetics and Ploidy 30 April 2014

- Manchester Insights Oct 2013

How to get a beautiful embryo

- Paris Ovarian Club Nov 2014

Laboratory aspects of time lapse imaging

- Catania UMR and HERA congress. July 2015

Details of embryo development in time lapse monitoring, definition and nomenclature of morphokinetic parameters.

- Manchester RHG International congress October 2015

Enhancing knowledge, improving results!

- Rome. Eurpoean Fertility Meeting. December 2015

Clinical value of embryo morphokinetic analysis

- Dundee. Scottish Embryologists' Meeting. June 2016

EmbryoScope annotation.

- Antalya. Society of Reproductive Medicine and Surgery congress. October 2017.

Utilisation of time lapse technology in the IVF lab: Pitfalls and benefits
Standardisation of the IVF lab – debate.

- Cairo. Upper Egypt congress. February 2018

Time lapse imaging and embryo selection. The CARE Fertility group experience.
Technical aspects of embryo biopsy and optimizing lab practice for PGS.

- Antalya. Turkish Society of Reproductive Medicine 2018

Embryo selection using time lapse monitoring.

- Reyjkavik. Alpha 12th biennial conference. May 2018.

Time Lapse. Useful algorithms or just nice videos?

- ESHRE May 2018. A decade of time lapse imaging: Reflections & future possibilities, ESHRE pre-congress course.

- London. COGI. 26th World Congress. November 2018

Useful algorithms or just nice videos?

- London. Royal College of Nursing Forum conference. March 2019
  EmbryoScope and the role of the embryologist

- Bologna. 9.baby symposium. October 2019.

Time lapse technology is here to stay. And for good reason!

- Lisbon. International forum for artificial intelligence in IVF. November 2019

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List of abbreviations

hCG – Human chorionic gonadotrophin
FSH – Follicle stimulating hormone
hMG- Human menopausal gonadotrophin
IVF – *in vitro* fertilisation
ICSI – intracytoplasmic sperm injection
HFEA – Human Fertilisation and Embryology Authority
aCGH – Array comprehensive genome amplification
NGS – Next generation sequencing
PGT-A – Preimplantation genetic screening
ERA – endometrial receptivity assay
WOI – Window of implantation
GnRH – Gonadotrophin releasing hormone

Thesis abstract

This thesis deals with a technology that is increasingly being incorporated into clinical IVF treatment with the aim of enhancing outcomes for sub-fertile patients: Time lapse imaging offers a less disturbed embryo culture environment than traditional incubation and selection methodologies, with the additional opportunity to time stamp and study, more precisely, *in vitro* developmental events, or 'morphokinetics'. Its relationship to preimplantation genetic testing for aneuploidy (PGT-A), which enables inference of embryo ploidy following embryo biopsy, and the preferential selection of euploid embryos, is much discussed in the context of time lapse morphokinetics - being considered as a non-invasive possible alternative to PGT-A. Both technologies have been widely, but not exclusively, reported to effectively select embryos from a cohort with the highest chance of success, and hence, by preferential selection and transfer - reducing the time taken for patients to attain a live birth.

This thesis considers the implementation of time lapse imaging in IVF laboratories; comparing approaches, impact and outcome measures of success. To meet its aims, this thesis incorporates a study designed to assess whether there is an association between the morphokinetics of the human preimplantation embryo in vitro, and its chromosomal complement, or ploidy. A novel algorithm is derived which, using morphokinetics, ranks embryos according to their risk of aneuploidy. The clinical relevance of this model is then assessed within this thesis and consideration is given as to how this non-invasive approach may enhance implantation and live birth rates in IVF. The thesis presents studies designed to look for novel morphokinetic markers of embryo viability with the development and use of objective algorithms to aid embryo selection, enhancing outcomes compared with conventional morphological selection. The thesis critically reviews a time lapse approach to

predict blastulation by use of multicentre outcome analysis, as part of a collaboration and goes on to present proposed standard guidelines for the nomenclature and annotation of dynamic human embryo monitoring, and IVF laboratory performance indicators, resulting from consensus collaborations with the international IVF scientific community.

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1.0 General Introduction

1.1 Infertility and IVF

The World Health Organization definition of infertility is "a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse" (Zegers-Hochschild *et al*., 2009). The incidence of Infertility or subfertility according to the National Institute of Health and (NIH) and European Society for Reproduction and Embryology (ESHRE) is around 1 in 6 of couples trying to conceive. https://medlineplus.gov/infertility.html

There are many causes of infertility that can include uterine factors, endometriosis, tubal factor, male factor, ovulatory disorders, luteal phase defects, repeated pregnancy loss, idiopathic, or advanced maternal age. Treatments to alleviate infertility range from simple timing of intercourse, to *in vitro* Fertilization (IVF). Simple assisted conception treatments, such as intrauterine (IUI) or intracervical insemination (ICI), may be applied to patients with blocked fallopian tubes, sufficient motile sperm available, normal hormone profiles and a functional uterus. Other simple interventions include timed intercourse, clomiphene citrate to stimulate follicle growth, hCG administration trigger ovulation or low dose gonadotropin administration to encourage ovarian follicular growth. Assisted Reproductive Technology (ART) treatments generally refers to where oocytes and embryos are handled outside of the body and more complex procedures are utilised.

This thesis is concerned with a specific type of ART i.e. *In vitro* Fertilization; IVF. The first reports of clinical IVF were famously Louise Brown and Alastair MacDonald in 1978 and 1979 respectively, and the technique has gradually evolved in the intervening years, making IVF a

routine treatment for subfertile patients. During 2017, around 75,000 IVF treatment cycles were performed within the UK. The recently published Human Fertilisation and Embryology Authority (HFEA) trends and figures report, of May 2019, showed the overall IVF birth rate per embryo transferred in 2017 to be 22% in the United Kingdom (UK). This has gradually increased since recording began in 1991 when this figure was only 7% (HFEA.gov.uk). The success of IVF can be broadly broken down into two key factors: the quality of the embryo and the quality of the uterus.

IVF involves hyper-stimulating the ovaries, in a controlled fashion, and the subsequent retrieval of around 10-20 oocytes. To achieve this, the pituitary gland is down-regulated by administration of a gonadotrophin releasing hormone (GnRH) analogue (agonist or antagonist) to suppress endogenous hormones before the cycle begins. Exogenous administration of follicle stimulating hormone (FSH) and/or human menopausal gonadotrophin (hMG) results in multiple follicular recruitment and development. As these follicles grow, they each produce oestrogen, which causes the endometrium to proliferate. At a follicle diameter of ~17mm an artificial luteinising hormone (LH) surge is most commonly created by administering human Chorionic Gonadotropin (hCG) or combination of high dose agonist/low dose hCG which has a similar effect as LH on oocyte maturation and ovulation. If an embryo transfer is to occur in the stimulated cycle then exogenous progesterone is administered post oocyte retrieval to prepare and maintain the endometrium. For fertilisation to occur, insemination takes place within the laboratory whereby sperm are either mixed with the oocytes (IVF), or injected (intracytoplasmic sperm injection (ICSI)) into each mature oocyte, on the day of oocyte retrieval. Following fertilisation assessment the

following day, the resultant embryos are kept in culture generally until the blastocyst stage of development.

Embryo transfer typically occurs on day 5 post fertilization, at the blastocyst stage and supernumerary blastocysts can be cryopreserved, by vitrification. Vitrified and warmed embryos can later be transferred in either a natural or artificial cycle. In a natural transfer cycle, transfer is timed 7 days post hCG trigger or 6 days post LH surge. In an artificial cycle down-regulation of the pituitary gland and administration of exogenous oestrogen can be used to prepare the endometrium. Once the thickness and pattern of the endometrium are appropriate, progesterone administration can begin.

1.2 Endometrial factors

Implantation is a complex process involving both embryo and endometrial factors. Gene expression shows marked changes during the cycle and, at the most receptive phase, growth factors, cytokines, glycoproteins and immunosuppressive agents are secreted into the uterine cavity (Melford *et al*., 2014). The most receptive phase of the cycle for the endometrium is called the "window of Implantation" (WOI); about 5-7 days post ovulation (Lessey, 2000). Embryo transfer timing during an IVF cycle aims to identify the WOI, which may differ between patients (Mahajan, 2015; Ruiz-Alonso *et al*., 2013). Commercial endometrial receptivity assays (ERA – Igenomix, or ER Peak – Cooper Surgical) have been developed by analyzing an array of genes differentially expressed at different stages of the menstrual cycle. An algorithm has been developed and implemented to predict a personalized WOI for patients with repeated implantation failure (Ruiz-Alonso *et al*., 2012) to capture the WOI. Gomez *et al* (Gomez *et al*., 2015) states that the ERA has shown that one in four patients with

repeated implantation failure have a displaced WOI and a recent randomised controlled trial, provides proof of principle evidence for the potential of using personalised diagnosis of the endometrial factors in the work up of the infertile couple, from the outset. This large study demonstrated a significant increase in cumulative pregnancy rate per intention to treat when endometrial receptivity was considered and addressed, compared with controls. (Simon *et al*, ASRM presentation 2019).

This thesis is however concerned more with the quality of the embryo and whether techniques can be developed to select the most viable embryo more reliably than using simple morphological observation.

1.3 Factors affecting embryo quality

Culturing and handling embryos *in vitro* inevitably exposes them to potential environmental, and mechanical stresses, which they otherwise would not have encountered *in vivo*. One of the key challenges since the original IVF in the 1970s has been to achieve a stress-free environment for IVF and embryo growth, as close to physiological conditions as possible (Swain, 2010; Swain *et al*., 2016). This consideration encompasses the culture media, the gaseous incubation environment, the laboratory consumables, pH and temperature.

Culture media in use today has been modified over the years but still is based loosely on mouse embryo culture media; a balanced salt solution (Chronopoulou and Harper, 2015). Culture media manufacturers rely on the readily available supply of mouse embryos to assess each batch of media as testing on human embryos is not practically possible (Quinn and Horstman, 1998). This standard practice has limitations, however, as mouse embryos and

human embryos are not equivalent in their needs and sensitivity (Ackerman *et al*., 1985; Ackerman *et al*., 1984). The assurance to the end user by testing media on a mouse model is only that the media has no obvious toxins and that the media is able to support growth of mouse embryos to the blastocyst stage.

The precise composition of commercially available culture media today are not generally published by culture media companies, however, they are basically a balanced salt solution incorporating varied concentrations of glucose, pyruvate, and lactate to facilitate embryo development past traditional developmental blocks (Gardner and Lane, 1996). The more modern formulations include amino acids, chelating agents (EDTA) and growth factors.

A review by Mantikou comparing the efficacy of different culture media concluded that overall there was no evidence to show one medium to be superior to the others. (Mantikou *et al*., 2013). However, some studies do show differences in clinical outcomes between media. For example, a randomized controlled trial (RCT) by Kleijkers and colleagues which showed an increase in the incidence of low birthweight offspring using once commercially available medium, compared with another (Kleijkers *et al*., 2016). Whether media constituents or culture environment generally affects the dynamics and rate of cell divisions and embryo development is not entirely clear at this stage (Ciray *et al*., 2012; Meseguer et al., 2012).

As culture conditions have improved due to media development, improved knowledge and more sophisticated reliable and stable incubators being available, the ability to culture embryos beyond day 3, through to day 5 has led to more blastocyst stage transfers which has

also enabled an element of "self-selection." Nevertheless, selecting the best possible embryo for implantation is a challenge for all IVF and a central aspect of this thesis.

1.4 Estimating embryo viability conventionally

Given that it is a primary aim to minimize stress to the embryo *in vitro*, the least invasive and most reliable assay possible is desirable in IVF. Preimplantation genetic testing for aneuploidy (PGT-A), with the possible exception of sampling culture media, is invasive, as it involves the removal, biopsy, of part of the embryo; which will be discussed in more detail later within this thesis. The simplest tool currently used by embryologists to choose the best embryo for implantation is visual inspection or embryo morphology. Very commonly in IVF, the best available embryo, in terms of appearance alone, is chosen from a cohort on day 2, 3 or 5 to be placed into the uterus.

1.4.1 Stage of embryo development and transfer

If an embryo has successfully developed beyond day 3 when the embryonic genome is activated (Niakan *et al*., 2012) and undergone blastulation, logic dictates, and practice demonstrates that is has a better chance of implantation. Blastocyst morphology and growth rate correlates to some extent with the chromosomal constitution of the embryo and this is central theme of this thesis. Failure to select a viable embryo for transfer will inevitably limit the chance of a positive outcome in a given treatment cycle. But with highly successful cryopreservation programmes available in most fertility clinics, it could be considered that simply transferring the patients' embryo consecutively (following vitrification and warming) will eventually have the same end result as selection to prioritise the embryos according to their expected potential to implant: At some point, the best embryo will be transferred.

However, to minimise the emotional burden on patients and in some cases financial burden too, identification and selection of the most viable embryo should be a priority. Sequential observations can help build up a picture of the quality, or viability potential of the embryo *in vitro* and the focus should shift from embryo selection being heavily weighted on the embryo's appearance at the point of selection for transfer and more account should be given to the embryo's short history; its timeline through and from fertilisation. And, as many of the developing embryo's morphological features are most accurately assessed in relation to time, the assessment of these should ideally be performed within designated, evidence-based time windows.

1.4.2 Non-invasive embryo assessment methods

There are several potential non-invasive methods for assessing embryo viability but morphological, and more recently morphokinetic, are the most accessible and established. Another potential non-invasive method of embryo selection is oxygen consumption by embryo respiration. This method has been used to compare oxygen consumption rates between implanting and non-implanting human embryos. Higher respiration rates were seen in transferred and frozen embryos compared to discarded embryos and among the embryos that were transferred, higher respiration rates were seen for implanting embryos than non-implanting embryos. Respiration rates performed close to the time of embryo transfer showed the strongest correlation with implantation potential (Tejera et al 2011).

Over the past 25 years, researchers in the field of assisted reproduction have identified a number of potential metabolic markers of embryo viability but most have proved inadequate for the selection of embryos for transfer or cryopreservation.

Prior to the implementation of time lapse imaging, several groups have studied pronuclear morphology in attempt to correlate this with embryo viability markers such as blastocyst formation potential, implantation potential and ploidy status (Gianaroli *et al*., 2003; Kattera and Chen, 2004; Scott *et al*., 2000; Zollner *et al*., 2002).

As an assessment was being made anyway, involving disruption from the protected culture environment, more detailed and opportunistic assessment of the pronuclei and nucleoli was considered. In one study, even numbers of nucleoli in each pro-nucleus distributed during pronuclear abbutal was reported to predict blastocyst formation with some accuracy, and a positive correlation with implantation was demonstrated (Scott et al., 2000). Arroyo's group, however, looked at pronuclear morphology and ploidy status of the embryo. They reported no correlation (Arroyo et al 2010). Gianaroli's group studied the position of pronuclei within the oocyte, the pattern of nucleoli and the angle of the pronuclei from the polar bodies. A correlation was found between complex aneuploidies, inferred by FISH, and chaotic arrangements of pronuclei and nucleoli. Euploidy could not be accurately predicted and such studies were limited by the frequency of observations of the pronuclei (Gianaroli *et al*., 2003). Several researchers, since the dawn of IVF, have studied the embryo's first cleavage and associated early cleavage with increased implantation potential (Fenwick *et al*., 2002; Lundin *et al*., 2001).

Time lapse imaging has enabled scientists to capture in real time the formation and dynamics of pronuclei and of early blastomere divisions, and to study the morphology and kinetics of the early preimplantation human embryos and their association with viability. A recent study found the relative difference in male and female pronuclear area, prior to their breakdown,

to be correlated with birth rates and proposed a method for preferential embryo selection (Otsuki et al 2019).

1.5 Time lapse imaging and morphokinetics

The central premise of this thesis is that human embryos *in vitro* are not equal in their capacity to make a viable pregnancy and that their particular pattern of growth; that can be monitored using time lapse imaging, can be predictive of developmental and viability potential. The observation of morphology over time (morphokinetics) as an indicator of embryonic competency is as old as clinical embryology itself but has become more sophisticated and clinically utilisable due to technological advancements (Payne et al 1997). The correlation between embryo morphology and implantation potential has been extensively demonstrated and documented but, this series of microscopic, manual assessments of morphology are increasingly being considered relatively poor indicators of an embryo's potential to result in a healthy live birth, even if selection takes place at the blastocyst stage (Gardner et al 2015).

Conventional light microscopical observations, unlike time lapse imaging, cannot allow the precise timings of mitoses, or the observation of anomalous cleavage events of the in vitro pre implantation embryo, to be recorded. Furthermore, without time lapse, transient characteristics such as multinucleation can easily be missed when embryos are limited to a single daily observation. Time-lapse imaging and analysis ensures that such phenomena are observed and recorded allowing embryo de-selection, where appropriate.

The wealth of detail that can be observed and information gleaned from such time lapse images is remarkable. Throughout the preimplantation embryo's development, from insemination to uterine transfer, new phenomena, observed using time lapse imaging and

analysis devices, are being defined and observed as the study of human preimplantation embryology takes on a new direction.

In contrast, conventional morphological embryo assessment typically consists of several, sequential, timed daily microscopic observations and ranking of them is commonly guided by professional body or consensus guidelines, some of which may be out-dated. Using such systems, the preimplantation cleavage stage embryo is assessed primarily on cell number and degree of cytoplasmic fragmentation, as well as developmental stage, with a demonstrated association with viability (Alpha and ESHRE SIG 2011, Cutting *at al* 2008). These swift embryo observations, however, employed to limit exposure to, and potential stress caused by, the assessment environment, provide limited information on developmental rates or patterns and little insight into the chromosomal complement of the embryo.

This established morphological approach to human preimplantation embryo assessment and selection has evolved in recent years with the introduction of time lapse monitoring, enabling the kinetics, and transient behaviours of embryo development to also be assessed; with reports of improved embryo selection and clinical outcomes, using this 'morphokinetic' information (Meseguer et al 2012).

1.5.1 The potential of time lapse imaging

Several time lapse incubation devices are now commercially available for the IVF laboratory. They are designed to capture images of the fertilising and developing embryos at regular intervals (commonly 5-20 minutes apart), throughout the *in vitro* culture period, avoiding the need to remove the embryos from the protected and optimised environment, for visual, microscopic assessment. The Embryoscope™ (Vitrolife, Sweden) was the first instrument to provide this relatively stable, 'uninterrupted' incubation - combined with internal microscopy, as an alternative to a standard IVF incubator. The introduction of such time lapse devices has resulted in a plethora of reports describing human preimplantation embryo development in greater detail, with descriptions of both its theoretical and demonstrable impact on embryo selection, and clinical outcomes (Rubio et al 2012, Meseguer et al 2011, Chamayou et al 2013).

Time lapse monitoring of the dynamics of embryo development, often in association with conventional qualitative morphological observations, provides detailed morphokinetic information on individual embryos. These data, consisting of timings, morphologies, irregularities and durations of embryo developmental events, can be retrospectively analysed against outcome measures, such as blastulation, implantation, embryo ploidy or healthy live birth. In turn, these data can also be used to derive predictive selection models, or algorithms, or simple preferential selection (or deselection) criteria, for prospective use in the IVF laboratory. Meseguer et al., in 2012, first reported a 20% increase in pregnancy rate using time lapse systems, compared with standard incubation, and attributed this improvement to the stable culture conditions of the time lapse device, and use of morphokinetic variables for embryo selection (Meseguer et al 2012). More recently, several large studies, and randomised

controlled trials have also reported improvements when time lapse algorithms were used for embryo selection, compared with morphology alone (Pribensky et al 2017).

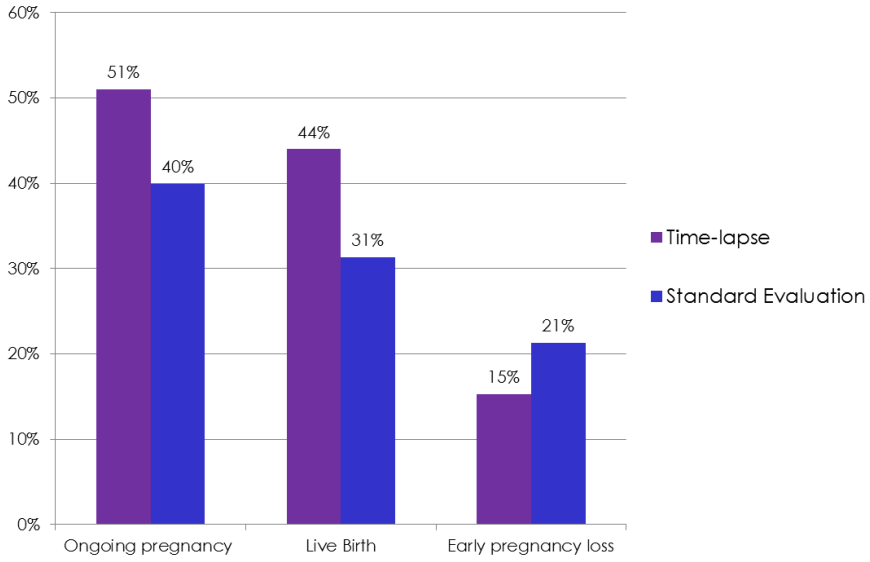

Figure 1: Summary of clinical results from meta analysis comparing the overall impact of time lapse and standard incubation and selection on clinical outcomes (Pribenszky *et al* 2017)

1.5.2 Cell cycles and nomenclature of time lapse imaging

A cell cycle is a series of complex events involving cellular and nuclear processes through particular phases that ensure the cell's division into two daughter cells. Mitosis consists of nuclear division and cytokinesis and several phases; prophase, prometaphase, metaphase, anaphase, and telophase. These are preceded by interphase which encompasses stages GAP-1 (G1), Synthesis (S), and GAP-2 (G2) of what is known as the cell cycle. The duration of cell cycles in the human pre implantation embryo appears to be related to embryo viability Ramsing and Callesen 2006). Prolonged or rapid cell cycles could be associated with DNA

repair, cellular rearrangements or failure of an embryo to undergo cell cycle checkpoints. Both potentially compromising embryo development (Ramos and De Boer 2011).

The first cell cycle following fertilisation, has been reported in detail following a large time-lapse study by Aguilar and colleagues (Aguilar et al 2013). In their study, they proposed some early time lapse terminology and described initiation of the first cell cycle as the time from the completion of second polar body extrusion. The length of S-phase (DNA replication) was defined as the time from appearance to fading of two pronuclei. In this study, embryos with prolonged S-phase demonstrated significantly reduced implantation rates compared with embryos with S-phases ranging from 5.7-13.8 hours. Completion of the first cell cycle can be defined as the time point that the embryo reaches the two-cell stage, such that the two daughter cells are discrete from each other.

Post-interphase cleavages of corresponding sister blastomeres rarely occur at precisely the same time and so time lapse users have considered the synchronisation of cell divisions within a round of mitosis, particularly as this has been reported to be correlated to embryo viability (Meseguer et al 2011). Synchrony of the second cell cycle would be defined as the duration of the transition of an embryo from two cells to four cells and calculated by subtracting the time the embryo reached the three cell stage (t3) from the time it reached the four cell stage (t4). With frequent time-lapse image acquisition (in some cases up to every minute), the duration of each cytokinesis can also be measured from the time a cleavage furrow is first visible to the time that the daughter blastomeres are discrete from each other (Wong et al 2010).

With time lapse imaging, cell cycle duration is calculated either according to a single cell division or as a round of mitosis whereby the number of blastomeres doubles. For the first cell cycle, as development begins with the single cell, these are the same. However, the second cell cycle begins with two cells, both of which should subsequently divide, forming two daughter cells each. There are therefore two individual blastomere cell cycles but a single embryo cell cycle which results in the doubling from two to four cells.

Although the nomenclature and annotation of time lapse variables are discussed in greater detail within the publications described throughout this thesis, the following schematic (figure 2) has been incorporated into the introduction in order to provide an overview as to how cell divisions and embryo development are referenced using this technology. The most frequently recorded morphokinetic variables follow the basic chronology of embryo development and mitosis, and include precise assessment of the timing of pronuclear appearance and fading, of increasing cell numbers, as well as assessment of fragmentation, cell evenness, synchronicity and nucleation, and the times to reach, and durations of, embryo differentiation to the morula and blastocyst stages.

Annotation practice and nomenclature are based on times (t) from insemination (IVF or ICSI) to pronuclear fading (tPNf) through each cell division to 8 cells (t2 to t8), morula (tM), start of blastulation (tSB) and the full blastocyst stage (tB).

Rounds of cell division are coded as embryo cell cycles ‘ECC’; when cell number doubles (e.g. from 4 to 8) and the blastomere cell cycles are coded as ‘cc’; the numbers representing sequential divisions and the letters, representing the individual blastomeres.

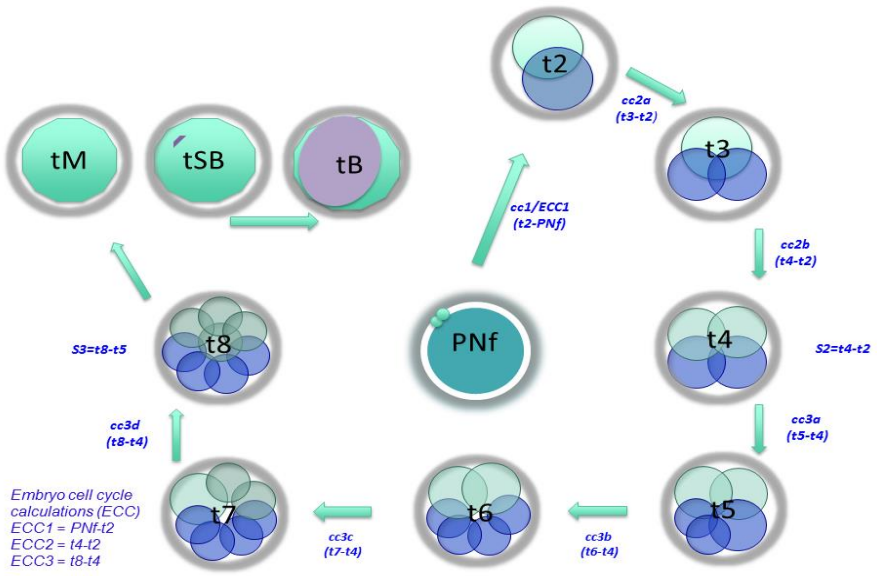

Figure 2: Schematic of standard morphokinetic variables

In: Campbell A, Fishel S, ed. Atlas of Time Lapse Embryology. Boca Raton, FL: CRC Press; 2015

1.5.3 Time lapse data collection and handling

Although automated, or semi-automated, time lapse video analysis and interpretation is likely to be increasingly used in the future, so far, data generated from these devices has primarily come from manual image assessment and recordings of the events observed (that is, by 'annotation'), on human review of the time lapse images viewed as videos. Due to the manual nature of data collection, to minimise subjectivity and maximise data quality, rigorous training of practitioners and quality assurance is required to assure the output and value of a clinical time lapse offering (Campbell et al 2013) (See appendix 3). The use of intra correlation coefficients enables assessment of different practitioners and reproducibility of annotations of the morphokinetic variables. Sundvall and colleagues considered inter and intra practitioner variation in annotation, using this intra-class correlation coefficient and demonstrated close correlation between experienced and newer time lapse users for most

morphokinetic variables, but highlighted that some 'static morphologic parameters' such as multinucleation and blastomere evenness, were at risk of subjectivity (Sundvall et al 2013).

Where there are multiple practitioners involved in time lapse data collection, the risk of subjectivity and inconsistency are enhanced, and intra-practitioner annotation variation may also exist. Minimisation of this subjectivity can be achieved by clear definition of key variables, and with strict annotation practice, set out within the standard operating procedure (SOP). Guidelines for nomenclature and annotation are available to encourage international consistency and to allow compilation of large data in the future (Ciray et al 2014).

Selection, for intra-uterine transfer, of the most viable embryo from a cohort in assisted reproduction technology (ART), is essential to minimise the time taken to reach pregnancy and successful delivery of a healthy child. With increasing female age being the most negatively impactful variable associated with fertility, failure to choose the best embryo first time could not only prolong the patient pathway and introduce emotional and financial burden, it could also reduce the chances of successful outcome during future attempts at IVF by delaying this process, as maternal age advances.

Time lapse (morphokinetic) analysis alone is considered by many to provide superior embryo selection to traditional and static morphology, and time lapse selection algorithms have been developed, published and independently evaluated, although reports of their performance, compared with traditional morphological selection, and their transferability between clinical settings is inconsistent, in part due to lack of standardisation and the heterogeneity in its application (Barrie 2017).

The morphokinetic data of a specific and transferred embryo which has a known outcome is commonly referred to being of 'known implantation data' or 'KID'. The known outcome may be a pregnancy test result (positive or negative), a gestational sac or foetal heart on ultrasound scan, a pregnancy loss or a live birth. Morphokinetic data can be compared between embryos giving positive or negative implantation data (KID+ or KID-), depending on the outcome measure used. All data can be utilised following a single embryo transfer, or a double embryo transfer with a negative outcome as the fate of these embryos is known. Using data following multiple embryo transfer that has resulted in the same number of foetal hearts or babies born may be problematic without the use of genetic fingerprinting to ascertain the chorionicity or zygoticity.

KID positive rates (or ratios) can be calculated for each morphokinetic variable in order to consider their impact and potential use in embryo selection models which may incorporate optimal time values or durations, to reach developmental milestones.

Recently, deep learning has been implemented in several areas of medicine. It has also been proposed as a superior, automated and standardised approach to facilitate embryo selection, using photographic data from time lapse imaging. A large multicentre retrospective analysis of over 10,000 embryos used a deep learning trained model to predict clinical pregnancy with a average area under the curve (AUC) of the receiver operating characteristic (ROC) curve of 0.93 (95% CI 0.92-0.94). Prospective studies are required in order to assess the potential clinical impact of this approach that promises to offer an automated method to improve identification of the embryo(s) from a cohort with the highest propensity for implantation

(Tran *et al*., 2019).

1.5.4 Selection and deselection morphokinetic criteria

In addition to enabling detailed, time-stamped monitoring of the morphokinetics of embryo development, time lapse technology also allows the identification of aberrant embryo cleavage events, which may not be observable using 'snap shot' traditional static microscopy methods. Potentially the most valuable example of aberrant cleavage, and one of the most well reported, in terms of its correlation with viability, is the phenomenon of a multi, as opposed to dichotomous, cleavage of a blastomere to three, or more daughter cells. Several studies have demonstrated the ability of time lapse to identify such aberrant cleavage divisions and highlight the reduced implantation potential of these embryos, compared with their counterparts that mitotically divide into two daughter cells. In a cohort of 1659 transferred embryos, the incidence of this 'direct division' (occurring within 5 hours) was 13.7% and the implantation rate of these embryos was markedly and statistically significantly lower than for embryos with a normal cleavage pattern (1.2% vs. 20.2% respectively (Rubio *et al*., 2012). Some investigations into this phenomenon have considered whether this erroneous cleavage phenomenon could be the cause or effect of aneuploidy within the cell (Zhan *et al*., 2016). Since the first reports of this phenomenon, the nomenclature of this common example of early erroneous cell division has been proposed, in an expert panel publication. An aberrant cleavage from a single cell directly to three daughter cells has been defined as trichotomous mitosis. This can be distinguished from 'rapid' cleavages, which appear as an accelerated division event, within 5 hours of the previous cell division (Ciray *et al* 2014). The hypotheses of Leese *et al*; that preimplantation embryos which are 'quiet', metabolically, have greater viability than those which are not, has recently been revisited

using retrospective and prospective data on metabolic and kinetic activity of preimplantation embryo development. The group considered that there may be optimal ranges which may influence embryogenesis. They proposed that there may be a "Goldilocks zone," within which embryos with maximum developmental potential can be categorized (Leese et al., 2016). This would sit well with what is being revealed from the increasing number of published time lapse studies.

Recently, researchers have searched for novel indicators of embryo viability, only accurately recorded using time lapse imaging. A large unpublished study at CARE Fertility examined close to 2500 blastocysts with known implantation outcome, or KID. In this study, logistic regression analysis demonstrated a significantly higher clinical pregnancy rate (48.9% versus 35.9%; $p<0.0001$) when morulae were fully compacted, with no excluded material, compacted to their counterparts.

1.5.5 Time lapse algorithms

Early adopters of time lapse and its implementation for embryo selection were Basile, Meseguer and colleagues, who used a hierarchical approach to time lapse algorithm development whereby embryos received a classification based primarily on reaching developmental milestones and the relative timings associated with them Basile *et al*., 2014). They reported significant differences between implanted and not implanted embryos for six early morphokinetic variables. The most significant, for implantation prediction, being the time that the embryo reached the 5 cell stage (t5), and the duration of the second cell cycle (cc2). The resulting selection model also included three exclusion criteria, based on their

negative association with implantation. These were rapid early division (from 2 to 3 cells), uneven blastomeres and multinucleation at the 4 cell stage. Since then, further time lapse models have been published with variation in both the time lapse variables of significance, outcome measures and the timings of specific developmental events (; Motato et al., 2016; Conaghan et al., 2013).

1.6 Preimplantation genetic testing for aneuploidy (PGT-A)

That human oocytes and embryos carry a significant amount of aneuploidy, and that aneuploidy is believed to be the single largest cause of embryo implantation failure and miscarriage has made it a prime candidate for analysis during IVF. (Hassold et al, 2007; Keskintepe et al 2007; Fragouli et al, 2011). Unlike most other embryo assessment approaches, aneuploidy has clear clinical relevance, may be considered binary in its relationship to biological significance, and is of high enough prevalence to make it a priority for screening. Logic determines that by detecting and eliminating aneuploid embryos each attempt at IVF should be more efficient; and in some cases, patients will receive important information about their gametes and embryos, helping them move forward in their fertility journey.

In 2008 the CARE Fertility Group and Bluegnome (Cambridge, UK) pioneered the development of preimplantation genetic testing using array comparative genome hybridisation (aCGH) for clinical use, first considering polar bodies, then blastomeres and trophectoderm tissue for the assessment of the cell's full chromosome complement (Fishel et al, 2010; Fishel et al, 2011). CARE Fertility Group considered polar body initially in the belief that if the oocyte is aneuploid the embryo will be; and early studies confirmed this, but recognising that paternally-

contributed and post-fertilisation mitotic aneuploidy will not be detected. More recently, with improved embryo culture and sensitive testing methodologies, the most commonly used, and effective method of PGT-A uses trophectoderm biopsy.

Preimplantation genetic testing for aneuploidy (PGT-A) is, like morphokinetic analysis, aimed at optimising embryo selection, but by identifying embryo ploidy and enabling preferential selection of a euploid embryo for intra uterine transfer. Aneuploidy is prevalent in human oocytes and embryos and its incidence increases with maternal age. Depending on the age of the female or circumstances of the couple, more than half of their embryos are likely to be aneuploid (Fragouli *et al*., 2011). The avoidance of selecting aneuploid embryos for transfer during IVF treatment is a clinical imperative and the method of choice for assessing embryo ploidy currently is PGT-A which provides chromosome copy number from the trophoblast cells of the blastocyst. Various technologies have been utilised, such as microarray based comparative genomic hybridisation (aCGH), single nucleotide polymorphism (SNP) arrays or quantitative polymerase chain reaction (PCR), and next generation sequencing (NGS), which have confirmed the high incidence of aneuploidy in human embryos with varying, but nonetheless mostly positive reports of its efficacy (Fragouli et al., 2006; Munne et al., 1993; Yang et al., 2012).

Identification of the most viable single embryo for transfer, has become increasingly important in recent years, driven by regulation (such as in the UK) and good clinical practice, to reduce the number of multiple pregnancies and births. Since 2010, with the first reported live birth after polar body biopsy and comprehensive chromosome screening, the

identification of euploidy has been reported to have improved outcomes in specific circumstances, such as advanced maternal age (Fishel et al., 2010; Stevens et al., 2012; Anderson et al., 2019).

Anderson compared PGT-A with unscreened treatments reported within the United States national data (SART 2016). They found that PGT-A, in their programme, outperformed the national average for live birth rate in all age groups. A recent multicentre randomised controlled trial (STAR), however, did not result in similar findings; surprising advocates of this technology, finding significant associations only for advanced maternal age (Munne et al., 2019). The need to constantly consider alternative technologies for the assessment of both aneuploidy and overall developmental potential is therefore paramount. Despite PGT-A being considered effective at identifying euploid embryos, and enhancing outcomes in some (but not all) studies, there are nonetheless limitations of this technology, in association with assisted reproduction. Blastocyst biopsy, used to remove a small number of cells for assessment requires training, specific equipment and technical expertise, and the testing; often off site, remains expensive and time-consuming. Furthermore, this invasive approach has raised ethical concerns and meets regulatory barriers in some countries (Harper *et al*., 2014).

1.6.1 Aneuploidy and preimplantation embryo development

Prior to the clinical availability of time lapse imaging, there had been reports of aneuploid embryos being delayed around the time of blastulation. In an early study of mosaicism, using fluorescence *in situ* hybridisation (FISH) in human blastocysts, Evsikov and Verlinsky proposed a process of selection against aneuploidy cells starting at the morula-blastocyst transition (Evsikov and Verlinsky 1998). They demonstrated that human blastocysts had a significantly lower degree of mosaicism than early cleavage stage embryos and postulated there being a threshold level of abnormal cells at which the whole embryo degenerated.

Alfarawati compared blastocyst qualitative morphology with ploidy and demonstrated a weak association between blastocyst morphology and aneuploidy (Alfarawati et al., 2011). This group also considered embryo developmental rates and reported an insignificant trend towards aneuploid embryos showing slower progression to the most advanced blastocyst stages; and that embryos with complex aneuploidy were most delayed. This study was performed without the benefit of time lapse technology however, and the findings were not clinically applicable as no clear cut-off time point was given for discrimination between complex aneuploid embryos and euploid ones. The time or frequency of blastocyst morphological assessment was not discussed in that publication.

The period leading up to blastulation is a period of intense cellular metabolic activity, gene activation, rapidly increasing cell division and differentiation. Cell division, and the mitotic process is a series of complex structural rearrangements involving the kinetochore - attachments to the microtubules, cohesion molecules - for the crucially precise separation of the chromosome to ensure correct alignment on the spindle - the spindle assembly complex

and many highly specialised proteins subject to precise gene expression (Gonen et al., 2012; Clift and Marston., 2011; Vogt et al 2008).

Although the cause of a possible temporal delay in aneuploid embryos compared to their euploid counterparts may be unclear, there exist error detection and repair systems within the cells to prevent aneuploidy (Nasmyth and Haering 2009). It is possible, therefore, that errors in individual cells at this stage of the rapidly developing embryo involve complex biochemical systems delaying karyo- and cytokinesis, which could result in the detectable delay in blastulation, particularly evident if time lapse imaging were used.

Both ploidy analysis by PGT-A and morphokinetc variable analysis from time lapse imaging are techniques that, individually, in some hands, have demonstrated, to have the potential to improve significantly the incidence of clinical pregnancy compared with traditional embryo culture and selection methods. Therefore, by comparison of the morphokinetics of euploid and aneuploid embryos over the preimplantation cleavage period, the key question arises regarding whether morphokinetics of euploid embryos differ from those of aneuploid embryos, and whether there could be synergies between these technologies in ART.

1.7. Is there an association between embryo ploidy and morphokinetics?

The first time-lapse report of an association between early human embryo development and ploidy was 2012. Chavez and colleagues demonstrated that early cleavage events, up to the 4-cell stage, were tightly clustered by timing in euploid human embryos, whereas those of aneuploid embryos were more diverse (Chavez et al., 2012). In this study, only 30% of aneuploid embryos displayed timings similar to the euploid cohort. This group used a small

dataset to predict embryonic euploidy with 100% sensitivity and 66% specificity. A larger study, by Basile, analysing the chromosomal content of 504 embryos by blastomere biopsy on day 3, and aCGH, proposed a logistic regression derived hierarchical model to subdivide embryos in to 4 categories (A to D) according to expected risk of aneuploidy (Basile et al., 2014). The algorithms consisted of the two morphokinetic variables; the time interval between 2 and 5 cells (>20.5 hours) and the duration of the third cleavage cycle (t5-t3) (11-18 hours). Embryos were categorised according to whether they developed within the ranges and use of this model resulted in a significant decrease in the percentage of normal embryos with each decreasing category (A, 35.9%, B, 26.4%, C, 12.1% and D 9.8%, P<0.001).

Several time-lapse studies have reported peri-compaction and blastulation delays in aneuploid, or embryos with low implantation potential, compared with euploid embryos, or those with high implantation potential: Minasi and colleagues, assessing a large cohort of 1730 blastocysts, asked whether there were correlations among ploidy status, morphology and time lapse kinetics. They reported that euploid blastocysts exhibited higher morphological quality inner cells mass and trophectoderm, and a shorter time to start blastulation, expansion and hatching compared with aneuploid ones (Minasi *et al*. 2016). More recently, Mumusoglu and colleagues, using a clustered data analysis, reported a low to moderate ability to predict euploidy when patient related factors and ovarian stimulation were considered. They reported a small number of late morphokinetic variables to be significantly different when comparing euploid and aneuploid embryo development. Later stage delays from t9 and through to expanded blastocyst stage was reported in the aneuploid cohort (Mumusoglu *et al*., 2017).

Time lapse studies considering and comparing the morphokinetics of euploid and aneuploid embryos are heterogenic in their design and approach. However, most have not identified an association between early cleavage stage morphokinetic markers and ploidy, suggesting that euploid and aneuploid embryos develop similarly up until around the time of compaction, or around the maternal to zygotic genomic transition.

A systematic review, in 2018, asking whether time lapse parameters predict embryo ploidy, considered 13 publications between 2012 and 2017. The review also highlighted the heterogeneity of studies published to date on this topic. The 13 studies varied in stage of embryo biopsy, clinical indications for PGT-A, embryo culture conditions, statistical approaches and outcome measures. The authors concluded that in most studies considering ploidy and morphokinetics, the intervals between cellular cleavages were of more relevance than cleavage timings in the selection of euploid embryos. It also stated that most of the studies with biopsy conducted at the blastocyst stage, reported significant differences between the morphokinetics of aneuploid and euploid embryos at later (peri-blastulation) stages, and did not find early parameters as predictors of embryo ploidy (Reignier et al., 2018). However, all researchers did not comprehensively compare durations between morphokinetic variables. The systematic review concluded that more large-scale studies are needed to elucidate fully the putative association between ploidy and morphokinetic parameters. Further publications have since supported, and rejected, the overall findings of this review. Desai et al assessed 767 biopsied blastocysts and reported that the presence of two or more dysmorphisms was associated with an overall lower euploidy rate, and that ploidy status correlated significantly with starting blastulation, expansion and the tEB to tSB

interval (Desai *et al*., 2018). Zhang *et al.*, however, did not find any morphokinetic variables to be associated with ploidy but did report that a combination of PGT-A and time lapse monitoring improved implantation rate and ongoing pregnancy rate for PGT-A (Zhang *et al*., 2017). The time lapse studies assessing morphokinetic markers of embryo ploidy are summarised in table 1 below.

First Author and year of publication	Time lapse embryo assessment period	Morphokinetic variables associated with ploidy status	Evidence for potential morphokinetic markers of embryo ploidy?
Chavez *et al* 2012	Early cleavage stage	cc2, s2	✓
Basile *et al* 2014	Early cleavage stage	t5-t2, t5, cc3	✓
Chawla *et al* 2015	Early cleavage stage	t5-t2, cc3	✓
Del Carmen Nogales *et al* 2017	Early cleavage stage	t5-t2, t3	✓ (high risk aneuploidies)
Campbell *et al* 2013 and thesis section 2	Blastocyst stage	tSC, tSB, tB	✓
Kramer *et al* 2014	Blastocyst stage	None found	×
Yang *et al* 2014	Blastocyst stage	None found	×
Rienzi *et al* 2015	Blastocyst stage	None found	×
Minasi *et al* 2016	Blastocyst stage	tSB, tB, tEB, tHB	✓
Patel *et al* 2016	Blastocyst stage	t5-t2, cc3	✓
Mumusoglu *et al* 2017	Blastocyst stage	t9, tM, tSB, tB, tEB	✓
Desai *et al* 2018	Blastocyst stage	>/= 2 early stage dysmorphisms tSB, tEB, tEB-tSB	✓
Zhang *et al* 2018	Blastocyst stage	None found	×

Table 1: Summary of studies assessing the relevance of morphokinetic variables to predict embryo ploidy

Whether pre embryonic genome activation events, such as cleavage morphokinetics prior to the 8-cell stage, or later morphokinetic information representing the activated embryonic genome will give the most reliable selection criteria needs further study. Most of the earlier time lapse publications focussed on events up to the five cell stage, and as a prognosticator for blastulation, or implantation, rather than live birth. Whilst it is recognised that maternal effects may mitigate against the survival of a potentially viable embryo, and not least because aneuploidy is the largest single cause of failure – live birth outcome, in relation to morphokinetics as a selection tool, is considered the gold standard outcome measure. Whilst early time lapse markers may be easier, and therefore more objectively interpreted and assessed during annotation (or automated image analysis) they may not be as reliably

representative of onward embryo development and potential following activation of the embryonic genome, as later morphokinetic variables.

This area of clinical research is challenging, not only due to the heterogeneity of patient populations and clinical approaches, but also because embryo ploidy is not binary, and mosaicism exists within the human embryo. Furthermore, there is increasing evidence to suggest that laboratory factors, such as humidity, culture media or oxygen tension, could impact embryo aneuploidy (Swain *et al*., 2019).

1.8 Are there synergies between time lapse imaging and PGT-A?

In jurisdictions where PGT-A is permissible and performed, the use of time lapse imaging may compliment this treatment, both practically and clinically. The time lapse incubation system minimises the need to remove the embryo culture dish from the protected and controlled incubation environment. This has general advantages but for PGT-A, time lapse can add particular value. Continuous embryo monitoring provides the embryologist, preparing to undertake the trophectoderm biopsy procedure, more information about the relative developmental stages of the cohort of embryos, in order to set and optimise the timing of this technically challenging microsurgical procedure. Without the facility of time lapse imaging, the embryologist would be required to remove the culture dish from the incubator in order to assess the blastocysts, and their suitability for biopsy, with little or no knowledge of where they actually are in the process of development; expansion or contraction, for example. This information may also be helpful to set patient expectations on the quality and number of embryos available. In addition, this morphokinetic information, in experienced hands could be utilised to prioritise blastocysts for biopsy, if the number was restricted due

to capacity or cost. There is also a case for ranking euploid embryos for transfer using morphokinetic information, particularly where validated algorithms are applied, when patients are fortunate to have multiple euploids available.

1.8.1 Patient perspectives and time lapse imaging

The impact for the IVF patient, of incorporating time lapse monitoring of embryo development, goes beyond clinical outcome. The additional information provided to patients by IVF professionals regarding their embryos' developmental patterns and timings, is more informative than standard methodology and being able to see the images generated from this technology may enhance patient understanding and acceptance of their subfertility. Patient feedback has indicated that time lapse technology positively aids their understanding of events inside the embryology laboratory. When asked, 82% of patients reported that they agreed or strongly agreed with the statement; 'The use of time lapse monitoring has improved our understanding of what happens within the IVF laboratory'. (Unpublished CARE Fertility data, n=363 patients) (Figure 4). The facility to download and view the video of their transferred embryo's first few days of development is also very well received.

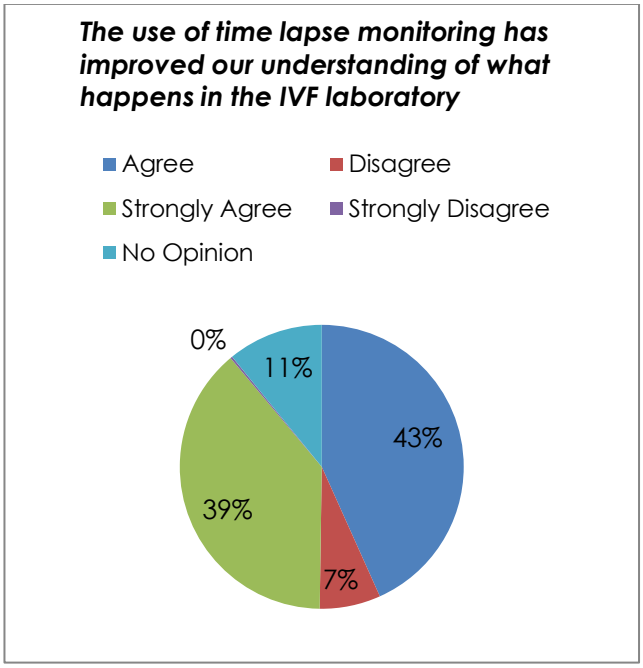

Figure 3: Pie chart to show patient feedback regarding the use of time lapse in their IVF treatment.

1.9 Perspective and thesis aims

From the literature, it is clear that time-lapse imaging in the context of improving IVF in general, and in terms of its relationship to aneuploidy, is a vibrant and continuously emerging field that could benefit many patients. The purpose of this study was to take a leading role in the development and application of time-lapse and morphokinetic analysis, and in educating the scientific community of its application and potential benefits. This was achieved through pursuit of the following specific aims, dealt with in 5 sections of this thesis:

1. To perform a retrospective time lapse analysis of euploid and aneuploid human embryos to identify any differences in their morphokinetic profiles and to develop a classification model to rank embryos according to their risk of aneuploidy.

2. To assess the clinical relevance of the aneuploidy risk model to aid non-invasive embryo selection to enhance implantation and live birth rates, without PGT-A.

3. To amass time lapse data during clinical IVF treatments to make novel observations and to develop in house embryo selection algorithms to predict implantation and live birth, and to compare the efficacy of such algorithms with conventional morphological selection.

4. To collaborate to perform a multicentre outcome analysis to consider the limitations of a time-lapse blastocyst prediction model.

5. To establish best laboratory practice for time lapse imaging, a) by engaging with the scientific community to develop and propose guidelines on the nomenclature and annotation

of dynamic human embryo monitoring; and b) by developing a resource for embryologists using time lapse imaging and c) by helping to develop international IVF laboratory performance indicators and IVF culture conditions in general.

2.0 Specific aim 1.

To perform a retrospective time lapse analysis of euploid and aneuploid human embryos to identify any differences in their morphokinetic profiles and to develop a classification model to rank embryos according to their risk of aneuploidy.

Encapsulated in this specific aim is the published study below which was the journal frontispiece, received runner up position in the Robert Edwards prized paper award 2013, was reported as the most cited article in RBMO in 2016 and received much media interest (See appendix 1).

Campbell A, Fishel S, Bowman N, Duffy S, Sedler M, Hickman CF. Modelling a risk classification of aneuploidy in human embryos using non-invasive morphokinetics. Reprod Biomed Online. 2013 May; 26(5):477-85. doi: 10.1016/j.rbmo.2013.02.006. Epub 2013 Feb 19. Erratum in: Reprod Biomed Online. 2013 Jul;27(1):107. PubMed PMID: 23518033.

In this study, my personal contribution was:

- Devising the operating procedure (SOP) for assessing and recording (annotating) the morphokinetic variables on human embryos in vitro using a time lapse incubation device (EmbryoScope). (See thesis section 6)
- Training of Embryologists to collect these data.
- Time lapse video annotation and laboratory work
- Data compilation and cleansing.
- Design of the study.
- Interpretation of statistical analyses.
- Manuscript preparation: writing of the first draft and amendments following review.

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ARTICLE

Modelling a risk classification of aneuploidy in human embryos using non-invasive morphokinetics

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Alison Campbell studied at Leicester and Nottingham Universities, specializing in assisted reproduction technology. She started her career as an embryologist in Liverpool and held a senior embryology position with CARE Fertility at its inception in 1997. Since then she has played a key role in establishing new laboratories and has headed up the embryology teams across the CARE organization. Her current role involves driving standards, best practice and leading research and development across the their laboratories in the UK and Ireland. Alison is an experienced clinical embryologist, a member of the HFEA licensed centres panel and a diplomate of the Royal College of Pathologists. Alison was responsible for the first clinical application of time-lapse microscopy in the UK.

Abstract This study determined whether morphokinetic variables between aneuploid and euploid embryos differ as a potential aid to select euploid embryos for transfer. Following insemination, EmbryoScope time-lapse images from 98 blastocysts were collected and analysed blinded to ploidy. The morphokinetic variables were retrospectively compared with ploidy, which was determined following trophectoderm biopsy and analysis by array comparative genomic hybridization or single-nucleotide polymorphic array. Multiple aneuploid embryos were delayed at the initiation of compaction (t_{SC}; median 85.1 hours post insemination (hpi); $P = 0.02$) and the time to reach full blastocyst stage (t_B; median 110.9 hpi, $P = 0.01$) compared with euploid embryos (t_{SC} median 79.7 hpi, t_B median 105.9 hpi). Embryos having single or multiple aneuploidy (median 103.4 hpi, $P = 0.004$ and 101.9 hpi, $P = 0.006$, respectively) had delayed initiation of blastulation compared with euploid embryos (median 95.1 hpi). No significant differences were observed in first or second cell-cycle length, synchrony of the second or third cell cycles, duration of blastulation, multinucleation at the 2-cell stage and irregular division patterns between euploid and aneuploid embryos. This non-invasive model for ploidy classification may be used to avoid selecting embryos with high risk of aneuploidy while selecting those with reduced risk.

KEYWORDS: aneuploid, embryo, EmbryoScope, morphokinetics, PGS, time-lapse monitoring

Introduction

Aneuploidy is prevalent in human oocytes and resulting embryos and its incidence increases with maternal age. Depending on the age of the female or the particular circumstance of the couple, more than half of their embryos are likely to be aneuploid (Fragouli and Wells, 2011). Avoiding the selection of aneuploid embryos for transfer after IVF is a clinical imperative. The only method available for assessing embryo ploidy uses invasive and expensive

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preimplantation genetic screening (PGS), which provides accurate full chromosome copy number of a biopsied blastomere from the early embryo, trophoblast cells of the blastocyst or the polar bodies of the oocyte and zygote. Various robust and reliable technologies have been recently introduced, such as array comparative genomic hybridization, single-nucleotide polymorphism arrays or quantitative PCR, which have confirmed the high incidence of aneuploidy in human oocytes and embryos (Fragouli et al., 2006, 2010; Kamiguchi et al., 1993; Munne et al., 1993). One of the main causal factors of aneuploidy is believed to be premature pre-division of chromatids and non-disjunction during meiosis, but several other contributory factors such as paternal or mitotic aneuploidy have been implicated. Aneuploidy in human embryos results in miscarriage, implantation failure or the birth of an affected child. PGS provides couples at high risk of embryo aneuploidy the opportunity for clinics to selectively transfer a euploid embryo, if available. Recently, it has been shown that the incidence of live birth in young, good-prognosis women, with a theoretically low risk of embryo aneuploidy is enhanced by PGS (Yang et al., 2012). Furthermore, where there is the need to transfer only a single or a maximum of two embryos, either by regulation (such as in the UK) or good practice, the selection of embryos from either euploid oocytes or embryos has improved outcome in specific circumstances (Fishel et al., 2010, 2011; Potter et al., 2012; Schoolcraft et al., 2012).

The development of commercially available time-lapse devices for the IVF laboratory during recent years has enabled embryologists to observe and study the development of human embryos continuously and, therefore, more precisely than was possible previously. Such monitoring of the dynamics of embryo development, in addition to traditional qualitative morphological observations, often referred to as morphokinetics, provides a plethora of information on the development of individual embryos. These data, generated by the manual or automatic recording or annotation of images collected over precise time points, can be retrospectively analysed against outcome variables, such as blastulation, implantation, ploidy or live birth in an attempt to identify prospective selection algorithms for embryo transfer. Morphokinetic analyses promise to provide IVF practitioners with novel markers of embryo viability. Recent studies (Dal Canto et al., 2012; Meseguer et al., 2011, 2012; Pribensky et al., 2010; Wong et al., 2010) have discussed the potential value of morphokinetic variables measured by time-lapse monitoring for improved embryo selection.

The application of time-lapse embryo monitoring in a clinical IVF setting avoids the need to remove embryos from incubation conditions to make a daily observation. It allows embryologists to rewind or freeze images in order to consider the detail and context of embryo development with practical flexibility. The EmbryoScope is the first instrument to provide a safe, stable incubation environment (with low incubation volume and direct heat transfer) combined with internal microscopy. Meseguer et al. (2012) observed a 20% increase in pregnancy rate compared with standard incubation and attributed this improvement to the EmbryoScope's stable culture conditions and use of morphokinetic variables for embryo selection. This study centre's experience is similar and this was the reason for the EmbryoScope being selected for this study.

As both ploidy analysis from PGS and morphokinetic variables from time-lapse imaging are two techniques that, individually, have been demonstrated to have the potential to significantly improve the incidence of clinical pregnancy compared with traditional culture and selection methods, this study examined whether euploid or aneuploid embryos display differing morphokinetic variables over the preimplantation cleavage period. Using trophectoderm biopsy subsequent to controlled culture conditions, the objective of this study was to determine whether embryos with a single or multiple aneuploidy displayed temporal morphokinetic variables that were significantly different from euploid embryos. If any variants were established, a further objective was to develop a model to categorize the risk of aneuploidy in embryos based on this non-invasive morphokinetic data.

Materials and methods

Data obtained for this research were obtained from the treatment of 25 couples attending an independent IVF clinic (CARE Fertility, Manchester, United Kingdom) from May 2011 to July 2012. All protocols complied with UK regulation (Human Fertilisation and Embryology Act, 1990, 2008). The study did not require ethical or institutional review board approval, having been performed according to previously validated procedures. This was a retrospective cohort study blinded to ploidy, with recording of embryo development using time-lapse technology (EmbryoScope; Unisense Fertilitech, Denmark) and strict adherence to annotation protocols for the variables defined in **Table 1**.

Criteria for patients for pre-genetic screening

The couples selected for this study requested or were recommended PGS due to a history of recurrent implantation failure following IVF (defined as more than two failed IVF attempts), recurrent miscarriage (defined as more than two spontaneous miscarriages), severe male factor infertility, previous aneuploidy or advanced female age (defined as $>$37 years). Female age ranged from 31 to 47 years (mean ± standard deviation 38.6 ± 3.6 years).

Ovarian stimulation

Pituitary suppression and ovarian stimulation was performed in 75% of patients with a gonadotrophin-releasing hormone agonist (Suprecur; 0.5 ml subcutaneously daily; Sanofi Aventis, UK) or antagonist for the remainder (Cetrotide; 0.25 mg daily; Merck Serono, UK). Ovarian stimulation was achieved using human menopausal gonadotophin (Menopur, Ferring, UK) and/or recombinant FSH (Gonal-F; Merck Serono), with doses ranging from 150 to 600 IU per day according to patient type and response. No differences were observed in ploidy for the stimulation regimens or doses (data not shown).

Table 1 Definition of variables used in the analysis.

	Definition
Morphokinetic parameters and multinucleation, annotated daily up to the point of trophectoderm biopsy	
$t_{PNfaded}$	Time when both pronuclei had faded
t_n	Time from insemination to completion of division to *n* cells
t_{SC}	Time from insemination to when the first cells of the embryo began to join together and compact (images could be rewound in order to establish the earliest signs of compaction)
t_M	Time from insemination to formation of a morula, where all the cells had undergone the compaction process and cell boundaries were unclear
t_{SB}	Time from insemination to start of blastulation, when the first signs of a cavity were visible (images could be rewound in order to establish the earliest signs of cavitation)
t_B	Time from insemination to formation of a full blastocyst; when the blastocoele filled the embryo with <10% increase in its diameter
t_{EB}	Time from insemination to expanded blastocyst; when the blastocyst had increased in diameter by more than 30% and the zona pellucida started to thin
t_{HB}	Time from insemination to hatching blastocyst, when trophectoderm herniation through the zona pellucida was first observed
MN2	Multinuclearity at the 2-cell stage
MN4	Multinuclearity at the 4-cell stage
Variables of duration, calculated from the morphokinetic parameters	
cc2	Time of second cell cycle (t_3-t_2), from 2 to 3 cells
cc3	Time of third cell cycle (t_5-t_3), from 3 to 5 cells
s2	Time of synchrony of the second cell cycle (t_4-t_3), from 2 to 4 cells
s3	Time of synchrony of the third cell cycle (t_8-t_5), from 4 to 8 cells
Blastulation	Time of blastulation, from start of blastulation to formation of a full blastocyst (t_B-t_{SB})
$1 \rightarrow 3$	Direct or rapid (<5 h) cleavage from 1 to 3 cells, referred to as irregular division pattern
$2 \rightarrow 5$	Direct or rapid (<5 h) cleavage from 2 to 5 cells, referred to as irregular division pattern

Oocyte retrieval, denudation and ICSI

With the female patient under sedation with a combination of propofol (Braun, Germany), fentanyl (Auden McKenzie, UK) and midazolam (Hamelyn, UK), transvaginal ultrasound-guided oocyte retrieval took place 36 h post human chorionic gonadotrophin injection (10,000 IU; Pregnyl; Organon, UK; or Ovitrelle; Merck Serono) or agonist trigger, using a dual lumen aspiration needle (Swemed; Vitrolife, UK) connected to a vacuum pump (Rocket Medical, UK). Oocyte–cumulus-complexes were recovered from follicular aspirates using a stereomicroscope in a class II hood with a heated stage, washed and cultured in Ferticult IVF medium (Fertipro, Belgium) at 5% carbon dioxide in air, 37.0°C, maximum humidity, for between 2 and 4 h before cumulus cell denudation with 15–20 IU/ml cumulase (Origio, Denmark) in the same medium and complete removal of the *coronae radiatae* with a 140 μm pipette (EZ Squeeze; Research Instruments, UK). Oocytes at the metaphase-II stage underwent insemination by intracytoplasmic sperm injection (ICSI).

Embryo culture and incubation

Following ICSI, oocytes were placed individually in microwells of EmbryoSlides (Unisense Fertilitech) in 25 μl Global IVF medium (LifeGlobal) supplemented with 10% dextran serum supplement (Irvine Scientific) and were overlaid with 1.4 ml mineral oil (Fertipro, Belgium) in the EmbryoScope. EmbryoSlides were prepared around the time of oocyte recovery with medium and oil that had equilibrated overnight.

Once loaded with the inseminated oocytes, EmbryoSlides were placed into the EmbryoScope time-lapse incubator at 37C in 5.5% CO_2, 5% O_2 and 89.5% N_2 for at least 5 days. Culture was temporarily interrupted on day 3 in order to re-fresh the medium. This was performed by removing 20 μl

of culture medium from each microwell and replacing it with fresh, pre-equilibrated medium. The built-in microscope was used to acquire images of each fertilized oocyte every 20 min through seven focal planes.

Trophectoderm biopsy and pre-implantation genetic screening

Of the blastocysts in this study, 69 (70.41%) of them underwent a series of three 4-μm diameter laser pulses (Saturn Active laser and Integra micromanipulator; Research Instruments) to breach the zona pellucida on day 3 of culture in order to facilitate trophectoderm herniation for biopsy. For the remaining 29 (29.59%) blastocysts, laser breaching was performed at the time of the biopsy procedure. No differences were observed in time from insemination to start of blastulation (t_{SB}) or time from insemination to formation of a full blastocyst (t_B) between embryos which underwent this procedure and those which did not.

The timing of the procedure and the stage of blastocyst development when trophectoderm biopsy was undertaken varied in this study as they were performed in a minimum number of cohorts to reduce disruption to the incubation and for efficient working practice. All biopsies were performed on blastocyst-stage embryos on day 5 or 6 post oocyte recovery. The minimum stage and quality criteria for selection of blastocysts for biopsy and vitrification in this study were embryos that were at least full blastocysts, with at least a fair, easily discernible inner cell mass and at least a few cells forming a loose trophectoderm (equivalent to stage 2 grade 2; Alpha and ESHRE, 2011a,b).

The time of hatching could not be reliably assessed in the embryos where a facilitative breach was made in the zona pellucida. Where observed, the time from insemination to start of blastulation was recorded for the first expansion episode only and only when expansion occurred prior to biopsy. The cycles of collapse and expansion, although observed in some of the blastocysts, were not recorded.

Up to three blastocysts per patient were biopsied in one dish in individually labelled 20-μl microdrops of G-MOPS buffered medium (Vitrolife, Sweden) using a 33–37-μm inner diameter blastomere biopsy micropipette (Humagen, Origio). Where required, the zona pellucida was breached using a series of 4-μm laser pulses and 5–10 trophoblast cells aspirated into the biopsy pipette. Stronger laser pulses of up to 15 μm were used with aspiration and mechanical pressure to remove the cells from the blastocyst. In compliance with UK regulation and good practice, witnessing was performed at every stage by manual witnessing or Matcher barcode electronic witnessing (IMT International). Time-lapse monitoring ceased for the particular blastocysts that were removed from the EmbryoScope for biopsy. Following biopsy, the biopsied cells were placed into 0.2 ml thin walled-tubes and sealed and frozen by placing them into a freezer at −20°C prior to genetic screening and the blastocysts vitrified (Kitazato; Hunter Scientific, UK).

Trophectoderm biopsies of 98 blastocysts were amplified and analysed by either whole-genome amplification and array comparative genomic hybridization ($n = 37$; Genesis Genetics Europe, Nottingham, UK) or by single-nucleotide polymorphism microarray ($n = 61$; Natera, California, USA). Embryos were categorized as 'single' or 'multiple' aneuploid depending on the number of chromosomes affected.

The method by which analysis of trophectoderm biopsies via single-nucleotide polymorphism microarray occurred has previously been described (Johnson et al., 2010a,b). Briefly, biopsies were lysed and heat inactivated prior to DNA amplification. In order to determine the source of aneuploidy, parental buccal swabs or blood samples were collected. Genomic DNA was isolated and the standard Infinium II protocol (Illumina, San Diego, CA, USA) was used for analysis of these samples. The genotype of the biopsy samples was determined using Infinium II genotyping microarrays (CytoSNP-12 chips). Chromosome imbalances were determined using a specific algorithm, described in Johnson et al. (2010b), which used the information gained from the parental genotype and the biopsy sample to determine both whole-chromosome imbalances and structural abnormalities, along with the parental source of the aberration.

The method by which analysis of trophectoderm biopsies via array comparative genomic hybridization occurred as has previously been described (Fishel et al., 2011). Briefly, DNA was amplified from the biopsied cells using the SurePlex DNA Amplification System (BlueGnome, Cambridge, UK) according to the manufacturer's instructions. Amplification was assessed by DNA gel electrophoresis and only samples generating amplified product were labelled. Test and control sample product and SureRef Male DNA (BlueGnome) were labelled with Cy3 and Cy5, respectively, using the BlueGnome fluorescent labelling system, according to the manufacturer's instructions. Labelled test and control sample and SureRef Male DNA were co-hybridized using 24Sure microarrays version 2 (BlueGnome). The resulting 24Sure microarrays were hybridized, washed and scanned according to the manufacturer's protocol. Scanned images were analysed and quantified and whole-chromosomal copy number ratios were reported using the Cytochip algorithm fixed settings in BlueFuse Multi Software (BlueGnome).

Evaluation of time-lapse images

Time-lapse images were collected for the duration of the culture period, to the point of biopsy and were used for the assessment of fertilization and embryo development. The time of insemination by ICSI was programmed into the EmbryoScope when the slide was loaded, as the time point midway through the ICSI procedure. The EmbryoViewer image analysis software was used to log and display the precise timing of developmental events as they were annotated by the embryologists studying the time-lapse images. **Table 1** outlines the definitions of morphokinetic variables that were recorded in this study of 2 pronuclei embryos up to the point of trophectoderm

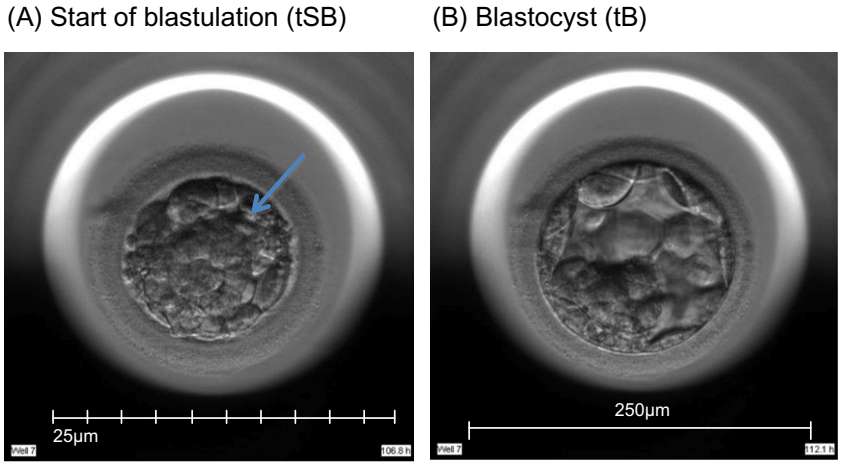

Figure 1 Time-lapse images of the same embryo demonstrating its appearance at specific time points as utilized in this study. (A) Human embryo at 106.8 hpi, with the start of a cavity forming (arrow), at t_{SB}. (B) Human blastocyst at 112.1 hpi, with a blastocoele filling the embryo with <10% increase in its diameter, at t_B.

biopsy. All times were recorded in hours post insemination (hpi). All annotations were made prior to the result of the biopsy analysis and were therefore blind. Example images show how the start and completion of blastulation were identified (**Figure 1**).

Data analysis

Embryos with incomplete annotations, failed amplification and/or abnormal or lack of fertilization were excluded from analysis. The time variables were tested for normality using Shapiro–Wilks test for normality. Since most variables were found not to be normally distributed, non-parametric tests were used to determine whether differences were significant. The Mann–Whitney–Wilcoxon test was used to test differences in morphokinetic variables, whilst Fisher's test (odds ratio different to one) was used to test differences in incidence rate of multinuclearity and irregular cleavage patterns. The statistical analyses were performed using R statistical software version 2.15.0 (R Foundation for Statistical Computing).

Algorithm using non-invasive morphokinetics to categorize the risk of aneuploidy

Morphokinetic variables found to differ between euploid and aneuploid embryos (single and multiple aneuploid combined) were used to build a decision tree model by recursive partitioning to partition the embryos into groups depending on the value of the variables t_{SB} and t_B. The recursive partitioning was optimized by the logWorth value. The partitioning process stopped when all new steps had a logWorth value below one (non-significant splits). The model classified embryos into three classes of aneuploidy risk: low, medium or high risk. The recursive partitioning and the probability of a random embryo in a particular risk class being aneuploid were calculated in JMP version 10.0 (SAS Institute).

Results

The time of initiation of compaction (t_{SC}) was significantly delayed ($P = 0.02$) for multiple aneuploid embryos (median 85.1 hpi, range 64.9–113.0 hpi) compared with euploid embryos (median 79.7 hpi, range 56.3–107.6 hpi). The time of initiation of blastulation (t_{SB}) was significantly delayed ($P = 0.004$ and 0.006) more than 6 h for both single aneuploid embryos (median 103.4, range 79.8–121.5 hpi) and multiple aneuploid embryos (median 101.9 hpi, range 86.8–129.4 hpi) compared with euploid embryos (median 95.1 hpi, range 85.2–113.9 hpi). The time of full blastulation (t_B) was significantly delayed ($P = 0.01$) for 5 h for multiple aneuploid embryos (median 110.9 hpi, range 90.1–137.0 hpi) compared with euploid embryos (median 105.9, range 86.8–122.3 hpi). All other timings tested were not significantly different (**Table 2**).

No significant differences were observed between aneuploid and euploid embryos in the length of the first or second cell cycle, synchrony of the second or third cell cycle, or the duration of blastulation (**Table 3**). No significant differences were observed between aneuploid and euploid embryos in multinucleation at the 2-cell stage or irregular division patterns (direct or rapid division defined as <5 h) cleavage from 1 → 3 or from 2 → 5 cells (**Table 4**).

Modelling

Since t_{SB} and t_B were found to differ significantly between euploid and aneuploidy embryos, these variables were selected for the simple classification model with three risk classes of aneuploidy (**Table 5** and **Figure 2**).

The following algorithm was derived using the recursive partitioning method in R: low risk, t_B <122.9 hpi and t_{SB} < 96.2 hpi; medium risk, t_B < 122.9 hpi and $t_{SB} \geq$ 96.2 hpi; high risk, $t_B \geq$ 122.9 hpi. When the data were divided into the three different classes the area under the receiver operating characteristic curve was 0.72.

Table 2 Timing of divisions for euploid, single aneuploid and multiple aneuploid embryos.

	Euploid				*Single aneuploid*					*Multiple aneuploid*				
	25th percentile (hpi)	*Median (hpi)*	*75th percentile (hpi)*	n	*25th percentile (hpi)*	*Median (hpi)*	*75th percentile (hpi)*	n	*P-value*	*25th percentile (hpi)*	*Median (hpi)*	*75th percentile (hpi)*	n	*P-value*
$t_{PNfaded}$	20.8	23.2	25.2	32	21.2	23.1	24.8	26	NS	20.8	22.6	23.9	30	NS
t_2	23.2	25.1	27.5	38	23.5	25.1	27.4	30	NS	23.3	24.9	26.9	30	0 NS
t_3	31.1	35.0	38.3	38	33.4	36.9	39.2	30	NS	33.2	35.6	37.4	30	NS
t_5	43.7	48.5	51.5	38	44.9	50.2	52.2	30	NS	43.9	47.7	50.6	30	NS
t_8	52.6	56.1	63.1	35	51.2	56.2	60.1	27	NS	48.8	54.0	60.3	27	NS
t_{SC}	74.1	79.7	85.4	35	75.3	80.7	86.7	30	NS	80.1	85.1	89.6	28	0.02*
t_M	79.6	83.5	87.8	32	80.5	87.9	93.6	29	NS	83.9	88.2	94.7	27	NS
t_{SB}	91.7	95.1	101.5	38	96.4	103.4	110.2	30	0.004*	97.0	101.9	107.3	30	0.006**
t_B	101.2	105.9	111.3	38	102.6	109.2	116.0	30	NS	105.9	110.9	116.3	29	0.01*
t_{EB}	104.5	110.6	114.0	18	105.3	109.2	113.3	13	NS	107.5	115.3	124.9	9	NS
t_{HB}	107.5	109.9	116.6	15	107.3	116.5	120.8	9	NS	114.5	115.4	118.4	6	NS

Mann–Whitney–Wilcoxon test, *P*-values of the two types of aneuploidy against the euploid: $^{*}P < 0.05$; $^{**}P < 0.01$.
hpi = hours post insemination; *n* = number of embryos: see **Table 1** for other definitions.

Table 3 Times of of developmental periods for euploid, single aneuploid and multiple aneuploid embryos.

	Euploid				*Single aneuploid*				*Multiple aneuploid*			
	25th percentile (h)	*Median (h)*	*75th percentile (h)*	n	*25th percentile (h)*	*Median (h)*	*75th percentile (h)*	n	*25th percentile (h)*	*Median (h)*	*75th percentile (h)*	n
cc2	9.8	10.7	11.8	38	10.3	11.4	11.9	30	9.4	11.3	12.0	30
cc3	10.8	12.5	14.0	38	11.7	12.6	13.7	30	11.3	12.3	14.3	30
s2	0	1.0	4.0	38	0	0.5	1.5	30	0	1.0	4.0	30
s3	2.6	8.7	15.9	35	3.0	6.7	10.4	27	2.5	4.0	13.9	27
Blastulation	6.8	8.3	11.0	38	3.6	6.9	10.9	30	5.3	9.7	12.3	29

Mann–Whitney–Wilcoxon test, *P*-values of the two types of aneuploidy against the euploid: No statistically significant differences were found ($P \geq 0.05$).
n = number of embryos; see **Table 1** for other definitions.

Table 4 Incidence rates of multinuclearity and irregular division patterns for euploid, single aneuploid and multiple aneuploid embryos.

Incidence (%)	*Euploid*		*Single aneuploid*		*Multiple aneuploid*	
	n	*Incidence (%)*	n	*Incidence (%)*	n	
MN2	13	38	16	30	10	30
MN4	0	38	0	30	0	30
$1 \rightarrow 3$	18	38	0	30	10	30
$2 \rightarrow 5$	2	38	3	30	13	30

Fisher's test for significant differences between the two types of aneuploidy against the euploid: no significant differences were found.
n = number of embryos; see **Table 1** for other definitions.

Table 5 A time-lapse derived model for the classification of ploidy with associated incidence rates and probabilities of aneuploidy.

Risk class	*Definition*	n	*Incidence*	*Probability*
Low	t_B <122.9 hpi and t_{SB} <96.2 hpi	36	0.36	0.37
Medium	t_B <122.9 hpi and t_{SB} ≥96.2 hpi	49	0.69	0.69
High	t_B ≥122.9 hpi	12	1.00	0.97
All		97	0.61	0.61

Corrected Akaike information criterion is 296; area under the receiver operating characteristic curve is 0.72; imputes = 1.
hpi = hours post insemination; *n* = number of embryos; incidence = incidence rate of aneuploidy embryos; probability = probability estimated using recursive partitioning-derived risk classification of an embryo being aneuploid; see **Table 1** for other definitions.

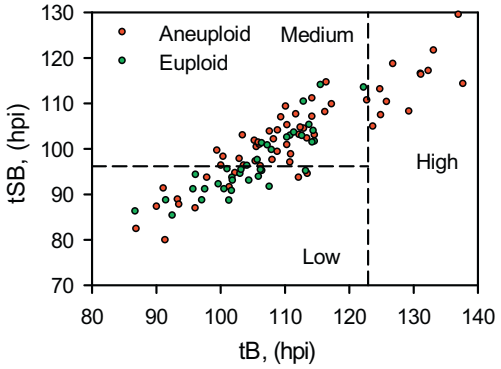

Figure 2 The three classes of aneuploidy risk (low, medium and high) based on time from insemination to a full blastocoele where the blastocyst has not yet started expansion (t_B) and time from insemination to start of blastulation (t_{SB}). Area under the receiver operating characteristic curve is 0.72. hpi = hours post insemination.

Discussion

As far as is known, this is the first paper demonstrating an association between human embryo ploidy and morphokinetics derived from time-lapse technology. The recent retrospective study by Meseguer et al. (2012), which set out to quantify the effect of using stable time-lapse culture conditions and morphokinetic variables for embryo selection, used clinical pregnancy as the end point. This was defined by the presence of gestational sacs with fetal heartbeat during week 7 of gestation. However, it is likely that a proportion of these early IVF pregnancies will be aneuploid. Fragouli and Wells (2011) discussed the landscape of blastocyst comprehensive cytogenetic analysis and reported overall aneuploidy rates in blastocysts to be greater than 50%. They suggest that the majority of biological selection against aneuploidy and genetic anomalies occurs at or after the time of implantation. It is widely accepted that a major cause of failed implantations or miscarriage following embryo transfer is the (unknowing) transfer of aneuploid embryos. Up until now, the only means available to IVF centres for assessing ploidy in embryos has been polar body, blastomere or trophoblast biopsy with PGS. Ploidy of the embryos in this study was assessed by screening of the trophectoderm to give full chromosome copy number, which is considered to give high concordance with the inner cell mass, but may still not be 100% accurate.

Alfarawati et al. (2011) previously compared blastocyst qualitative morphology with ploidy and demonstrated only a weak association between blastocyst morphology and aneuploidy. They reported that, concerning the growth rate of blastocysts, there was an insignificant trend toward aneuploid embryos showing slower progression to the most advanced blastocyst stages and that embryos with complex aneuploidy were most delayed. This study was performed without the benefit of time-lapse technology and the

findings were not clinically applicable as no clear cut-off time point was given for discrimination between complex aneuploid embryos and euploid ones. The time or frequency of blastocyst morphological assessment was not discussed in that paper.

From the cohort of blastocysts that underwent PGS in this study, the overall incidence rate of aneuploidy was 61%. The model used the morphokinetic variables t_{SB} and t_B of these embryos of known ploidy to classify a blastocyst's risk of aneuploidy. The model can therefore be used to rank individual and unscreened blastocysts as having low (probability 0.37), medium (probability 0.69) or high risk (probability 0.97) of aneuploidy. Especially where there is a choice of embryos available, the model could be useful in reducing the chance of selecting an aneuploid embryo. The classification from this model may, therefore, be used clinically to rank embryos for transfer and could also be used alongside PGS in order to prioritize embryos for biopsy and screening, particularly where costs are based on the number of embryos screened. Additional data may facilitate the fine tuning of this algorithm.

Embryos with rapid cleavage from 2 to 3 cells in less than 5 h have been reported to have a significantly lower implantation potential than embryos with a normal cell cycle length (Rubio et al., 2012). The normal cell cycle time has been established to be 10–12 h (Cummins et al., 1986) and therefore there is sufficient time for DNA replication. It is unknown if a causal relationship to ploidy exists in all cases of such anomalous cleavage: whether rapid cleavage provides insufficient time for complete genome replication resulting in aneuploidy or whether a particular existing aneuploidy results in erroneous cleavage patterning. For example, some aneuploidies are lethal during the preimplantation stage, such as many monosomies, whilst some, such as trisomies 13, 18 and 21, are compatible with full-term delivery. Somfai et al. (2010) reported a high frequency of chromosomal anomalies in bovine embryos which developed from zygotes that exhibited direct division to three from one cell, although rapid cleavage was observed in a small proportion of both euploid (18%) and aneuploid embryos (5% overall) in the present study. The incidence of such rapid cleavage was not assessed in embryos with arrested development in this study and further research into such cleavage patterning, such as reverse cleavage, is underway.

This study developed a model based on this study centre's own culture systems, in which all controllable environmental factors have been standardized and with a strict annotation policy followed by laboratory staff. This generated two precise time points, the start of blastulation and the time that the embryo reached the full blastocyst stage, as important markers in this model for classifying the risk of ploidy. With standard incubation, embryologists will be unable to annotate these timings precisely without tremendous disruption and compromise to incubation and working practice. Whilst a daily observational procedure could be applied on day 4 at around the same time of day as the insemination occurred (~96 hpi) and another on day 5 at a similar time (120 hpi) in an attempt to align with the temporal values in this model, the facility to study and rewind time-lapse images to identify the point of blastulation initiation (t_{SB}) and completion of blastulation (t_B) is essential to this modelling to permit a high degree of confidence in this data. Furthermore, the accuracy of the model is dependent on the 20-min imaging interval (i.e. capable of generating 19 images in 6 h) It is unknown at this time whether specific time points from this model can be applied to a different culture system, culture media, plastic ware, gas mix, air purity, etc., even with time-lapse technology.

This model for categorizing the risk of aneuploidy using non-invasive methods should improve clinical outcome. Other systems that simply predict the occurrence of an embryo to undergo blastulation (Wong et al., 2010) are unlikely to be as clinically robust given that all aneuploid pregnancies, miscarriages and live births, *ipso facto*, developed to and beyond the blastocyst stage, as did the many aneuploid blastocysts that failed to become pregnancies. The high incidence of aneuploidy in blastocysts (61% in this study), confirms the clinical limitations of time-lapse systems used only to select embryos that are predicted to become blastocysts.

The period leading up to blastulation is a period of intense cellular metabolic activity, gene activation, rapidly increasing cell division and differentiation. Cell division and the mitotic process is a series of complex structural rearrangements involving the kinetochore attachments to the microtubules (Gonen et al., 2012), cohesion molecules for the crucially precise separation of the chromosome to ensure correct alignment on the spindle (Clift and Marston, 2011), the spindle assembly complex and many highly specialized proteins subject to precise gene expression (Vogt et al., 2008). Although the cause of a temporal delay in aneuploid embryos compared with their euploid counterparts is not yet fully explained, there exist error detection and repair systems within the cell to prevent aneuploidy (Nasmyth and Haering, 2009). It is highly probable, therefore, that mitotic errors in individual cells at this stage of the rapidly developing embryo involve complex biochemical systems delaying karyo- and cytokinesis, which result in the gross observation of delayed blastulation.

Time-lapse photography in a closed incubation system such as the EmbryoScope, when used precisely to measure the initiation and completion of blastulation, appears to be the most reliable non-invasive method of ranking the risk of aneuploidy in advanced-stage embryos. It is unlikely ever to be as absolute as gaining accurate chromosome copy number from biopsied cells, but it is an important tool to enhance the chances of a live birth following IVF by non-invasive means. Further studies are underway to quantify further morphokinetic variables, to observe the impact of specific aneuploidy data and to prospectively test this model.

This study indicates that, with time-lapse monitoring of embryo development to blastocyst in a closed incubation system, it is possible, where there are alternative embryos available within a cohort, to avoid the selection of embryos having a high risk of aneuploidy and to preferentially select embryos with a greatly reduced risk of aneuploidy based on morphokinetic timing.

This non-invasive approach may be offered to patients as an alternative to PGS or, indeed, as a complementary system. This model for classifying the risk of aneuploidy requires time-lapse technology to enable identification of specific time points for embryo development up to the blastocyst stage. This accessible and non-invasive embryo

selection model may be used for patients electing against invasive genetic screening technology or for clinics without the skills or access to PGS.

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3.0 Specific aim 2.

To assess clinical relevance of the aneuploidy risk model to aid non-invasive embryo selection to enhance implantation and live birth rates, without PGT-A.

Encapsulated in this specific aim is the published study below plus an author response to a criticism of the paper.

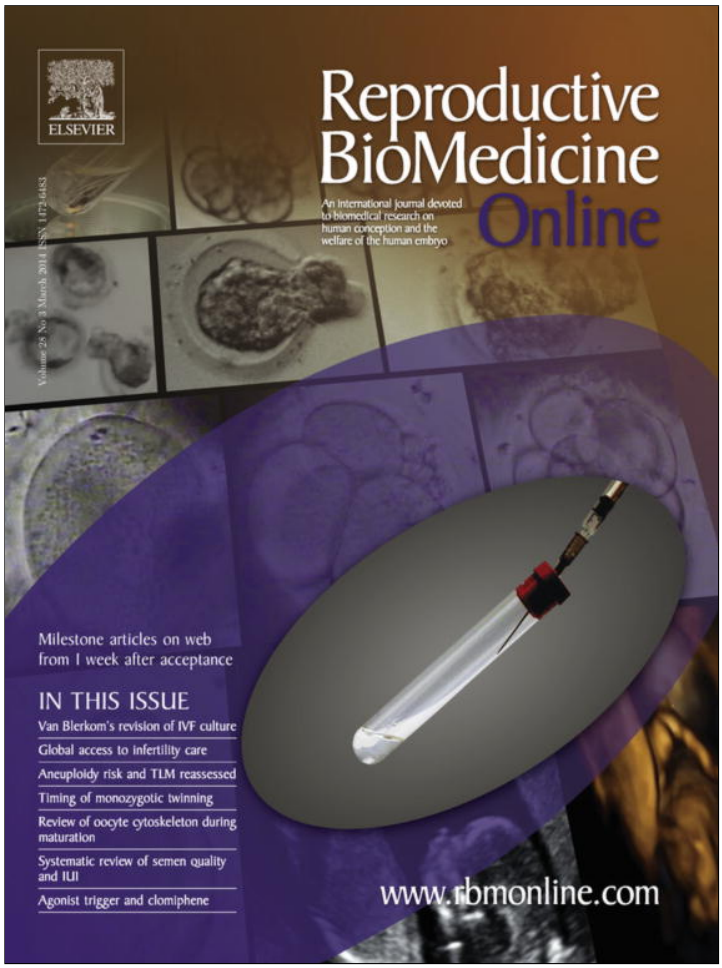
***Campbell A**, Fishel S, Bowman N, Duffy S, Sedler M, Thornton S. Retrospective analysis of outcomes after IVF using an aneuploidy risk model derived from time-lapse imaging without PGS. Reprod Biomed Online. 2013 Aug;27(2):140-6. doi: 10.1016/j.rbmo.2013.04.013. Epub 2013 May 9. PubMed PMID: 23683847.*

This publication received a certificate for 'Highly cited research' in Reproductive BioMedicine Online, November 2016

***Campbell A**, Fishel S, Laegdsmand M. Aneuploidy is a key causal factor of delays in blastulation: author response to 'A cautionary note against aneuploidy risk assessment using time-lapse imaging'. Reprod Biomed Online. 2014 Mar;28(3):279-83. doi: 10.1016/j.rbmo.2013.11.016. Epub 2013 Dec 11. PubMed PMID:24444816.*

In these studies, my personal contribution was:

- Study design and data preparation
- Interpretation of statistical analyses.
- Manuscript preparation: writing of the first draft and amendments following review.
- Preparation of response to criticism of the manuscript using further statistical analyses.



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COMMENTARY

Aneuploidy is a key causal factor of delays in blastulation: author response to 'A cautionary note against aneuploidy risk assessment using time-lapse imaging'

Alison Campbell [a,*], Simon Fishel [a], Mette Laegdsmand [b]

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Abstract In a previous paper, we had reported use of time-lapse monitoring to develop an aneuploidy risk classification model after identifying significant periblastulation delays in aneuploid embryos compared with euploid embryos. The model was validated subsequently in a second paper by retrospective assessment of transferred blastocysts that had also undergone time-lapse monitoring in which clinical pregnancy or live birth outcomes were established. A significant difference was seen for both outcome measures between embryos classified as low and medium risk by the model. Here we respond to the commentary entitled 'A cautionary note against embryo aneuploidy risk assessment using time-lapse imaging', which presented a case for our conclusions being unsound on the basis that maternal age, rather than aneuploidy, might be the cause of the developmental delays observed. We demonstrate that this is not the case and strengthen the argument that ploidy is a key factor influencing morphokinetics of blastulation. We also describe why the arguments made in the commentary based on comparisons between static standard observations and timings of the preimplantation embryo compared with those obtained from dynamic or time-lapse methodologies are inexact.

KEYWORDS: age, blastulation, IVF, morphokinetics, ploidy, time lapse

In the commentary 'A cautionary note against embryo aneuploidy risk assessment using time-lapse imaging', Ottolini et al. (2013) provide no information on their method or frequency of observations used, and so we must stress the shortcomings of describing an embryo as 'day 5' or 'day 6' when disputing the validity of a time-lapse study which records images at a rate of at least three times per hour throughout the in-vitro culture period. The delays that were reported and used to develop the aneuploidy risk classification model could not be detected without time-lapse analysis, nor can they be translated to standard practice (Campbell et al., 2013a).

Ottolini et al. (2013) reference the work of Kroener et al. (2012), suggesting that it 'clearly showed that delayed blastulation was not associated with increased aneuploidy rates'; however, this study did not use time lapse to measure precise timings and is confounded by the fact that it utilized blastomere biopsy for ploidy assessment which in itself has been demonstrated to affect subsequent embryo development, possibly delaying compaction (Kirkegaard et al., 2012). Furthermore, Alfarawati et al. (2011), using trophectoderm biopsy but also standard embryo assessment methods at unspecified time or frequency, reported a trend towards aneuploid embryos, showing slower progression to the most advanced blastocyst stages. Interestingly, the 'unpublished observations' cited by Ottolini et al. (2013) also represent a similar trend; the incidence of aneuploidy, on 'day 6' was 60.2%, compared with 53.4% on 'day 5'.

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Since the publication of our articles under discussion, Hong et al. (2013) have reported a significant correlation between early times to cavitation, from the first cytokinesis and from 5 cells, and reduced prevalence of aneuploidy.

Aneuploidy is prevalent in human oocytes and resulting embryos and it has been widely demonstrated that its incidence increases with maternal age. Ottolini et al. (2013) argue that it is maternal age, and not embryo aneuploidy specifically, which causes the delays described in our articles.

We respectfully suggest that Ottolini et al. (2013) misrepresent the reported impact of maternal age on blastocyst development by stating that it is 'well established that blastocyst formation and hatching are negatively correlated with maternal age' (Porter et al., 2002). Porter's study, on supernumerary blastocysts, demonstrated a statistically insignificant negative correlation between blastocyst formation and hatching and increasing maternal age. To our knowledge, there are limited studies that consider age and morphokinetics. Hickman et al. (2013) demonstrated in 348 embryos that maternal age did not affect morphokinetic timings up to the morula stage but that in women aged $\leq$36 years, embryos reached the later developmental stages significantly faster than in the those >36 years.

We examined over 1200 individual embryos, monitored by time lapse to the blastocyst stage, combined from four of our centres, where embryo culture and laboratory practice is aligned, to correlate maternal age with the morphokinetic variables used in our published model (Campbell et al., 2013a). These variables were the start of blastulation (t_{SB}) and the time to reach the blastocyst stage (t_B). The embryos were all from cycles with an autologous oocyte source.

Figure 1 confirms a positive but not a close correlation between age and both t_B and t_{SB}, which could lead to the assumption that age is controlling these morphokinetic variables. However, it is apparent that other factors are influencing these variables, as data are scattered, with the 95% prediction interval being around 20 h on either side of the trend or linear regression lines. The mean increase in t_{SB} over the range of maternal ages (25—45 years) is 5 h, whereas the interval where 95% of any future embryos are expected to fall (95% prediction interval) for a specific age is ±20 h. A similar pattern is seen for t_B. Age alone cannot account for these differences.

The question remains: does maternal age directly influence embryo morphokinetics or is it the differing proportions of competent (such as euploid) embryos which are controlling the apparent correlation between age and the average timing of late morphological events? Our work would suggest that embryo ploidy status is a key factor controlling these morphokinetics. Due to this, a prediction of the relative risk of aneuploidy and also of the relative chance of implantation can be calculated by the model we have described (Campbell et al., 2013a).

The model has since been further validated by Unisense FertiliTech on independent implantation data from 27 clinics under an agreement with Unisense FertiliTech (unpublished data). This analysis was based on 536 embryos with implantation information. The results are in concordance with our findings, indicating validity of the proposed model and supporting the practical usability of the model to assist

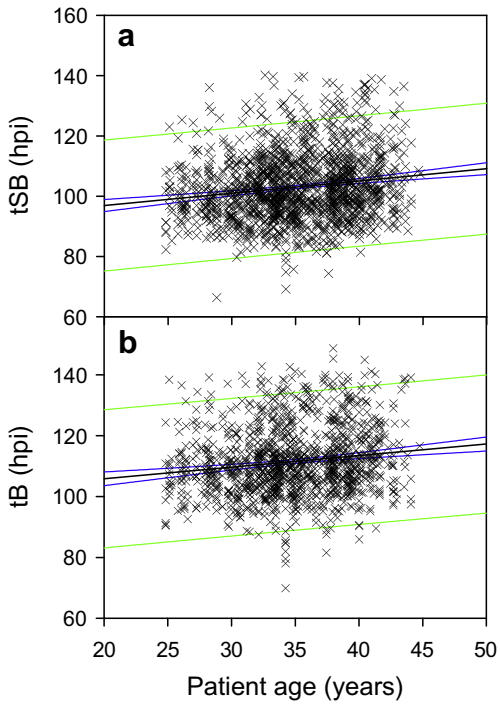

Figure 1 The time to start of blastulation (A, n = 1517) and to full blastocyst (B, n = 1271) in hours versus the age in years of the patients at the time of autologous oocyte retrieval. The black line is the trend (linear regression) line, the blue lines indicate the 95% confidence interval and the green lines indicate the 95% prediction interval. hpi = hours post insemination; t_B = time from insemination to a full blastocoele where the blastocysts has not yet started expansion; t_{SB} = time from insemination to start of blastulation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in the embryo selection process. Blastocysts categorized as having the lowest risk of aneuploidy by retrospective application of the model gave the highest clinical pregnancy rate, which was demonstrated to decrease as aneuploidy risk increased, by class.

Ottolini et al. (2013) accept that we have identified developmental time points which relate to an embryo's implantation potential in our system and we would suggest, in light of increasing evidence, that these two variables may also have relevance in other settings.

The ultimate embryo selection model, however, will use healthy euploid live birth as the outcome measure, rather than clinical pregnancy or ploidy status, as the absolute accuracy of the latter might be limited by trophoblast mosaicism. Implantation failure and biochemical pregnancy or miscarriage may also confound morphokinetic studies.

Ottolini et al. (2013) argue that the original analysis of 97 embryos is underpowered (Campbell et al., 2013a). In the original work, two statistical methods were applied to the data: (i) the difference in timings were tested between the euploid, single aneuploid and multiple aneuploid embryos; and (ii) the area under the curve (AUC) and the receiver operating characteristic (ROC) curve, were used as a measure of the classification power of the model. For the relevant variables (t_{SB} and t_B), the tests of differences

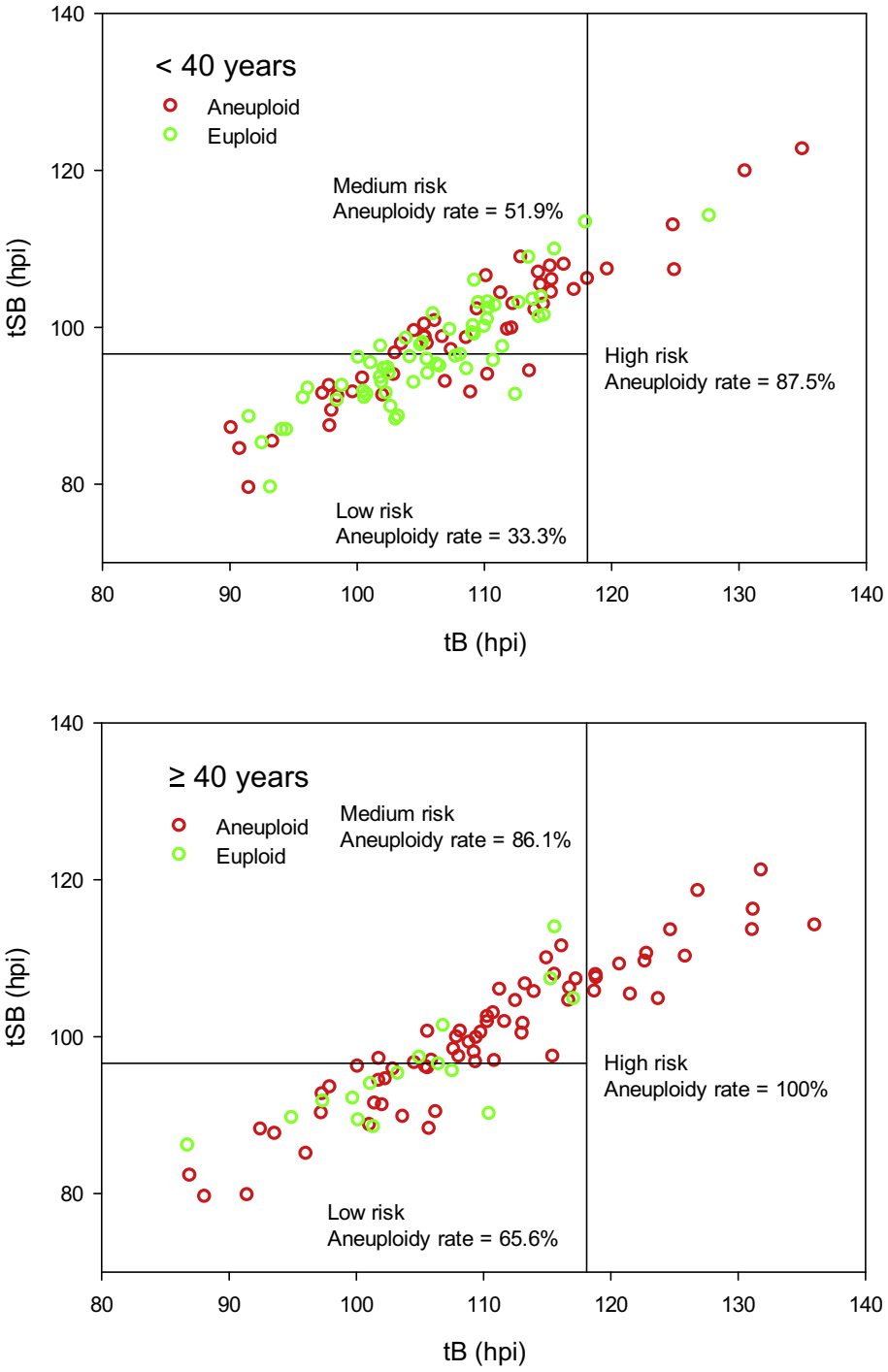

Figure 2 Three classes of aneuploidy risk (low, medium and high) based on time from insemination to a full blastocoele where the blastocysts has not yet started expansion (t_B) and time from insemination to start of blastulation (t_{SB}) with revised limits. Data are divided into patient age at the time of oocyte retrieval — <40 (A) and ≥40 years (B) — demonstrating how the risk classification model is effectively applied for patients in these two different age categories. hpi = hours post insemination.

were significant, with power between 0.96 and 0.98 ($P < 0.05$; Hintze, 2013). AUC, which was 0.72 in the original work, was significantly different from 0.5 (which is the no-effect value of AUC) with power 0.97 ($P < 0.05$; Hintze, 2013).

Ottolini et al. (2013) question whether it is necessary to include both t_{SB} and t_B in the aneuploidy risk classification model due to the two variables being apparently linked. The method applied in the original work was to make a recursive partitioning model with the variables that had proven to be significantly different between euploid and aneuploid embryos and only to include significant splits. Whist statistically it may be effective to utilize only a single variable (in this case the strongest being t_{SB}), from a biological perspective we elected to incorporate t_B into the recursive partitioning model as it is important to distinguish

Table 1 Logistic regression analysis of the combined effect of t_{SB} and age on aneuploidy.

Model effect	*Estimate (logOR)*	*Significance of additional effect*	*AIC* if removing single effect (full model = 238)*
Intercept	−11 (3)	<0.0001	
Age (continuous)	0.16 (0.04)	<0.0001	254
t_{SB} (continuous)	0.05 (0.02)	0.016	242

*AIC = Aikake information criterion.

between embryos which initiated blastulation but either had a very extended blastulation phase or did not reach the blastocyst stage (t_B) at all. Additionally, the pool of data that are allocated to the high-risk group could not be as effectively isolated using t_{SB}, since t_{SB} for most of the data in the high-risk group is equivalent to the medium range with regard to t_{SB}.

Since the publication of our work earlier this year, we have doubled the size of the dataset to 195 embryos allowing for fine tuning of the aneuploidy risk classification. A new analysis has moved the boundary for t_B in the high-risk class from 122.9 to 118.1 h post insemination and the boundary for t_{SB} between the low- and medium-risk classes from 96.2 to 96.6 h post insemination. The aneuploidy rate for the updated low-, medium- and high-risk classes is 45.8%, 65.9% and 95.8%, respectively. The AUC of the ROC curve is 0.67, which is significantly different from 0.5 with a power of 0.99 ($P < 0.05$) (Hintze, 2013). The difference between the medium- and low-risk groups were significant ($P = 0.01$) and the power was 0.80 ($P < 0.05$).

The revised model has been further split by two age groups: <40 and ≥40 years (**Figure 2**). The incidence rate of aneuploidy of each group gives reason to be confident in the ranking power of the model even with narrower age categories. It also demonstrates that the levels of aneuploidies may vary greatly in different age groups, underlining that the aneuploidy risk classification model should not be used to determine the actual risk of aneuploidy (e.g. 65.6% chance of aneuploidies) but to assess the relative risk of aneuploidies (e.g. embryo X has a greater risk of being aneuploid compared with embryo Y).

In response to the comments by Ottolini et al. (2013), we applied a logistic regression model to our data. **Table 1** shows a combined model for the effect of t_{SB} (the strongest variable in the aneuploidy risk classification model) and age. The required number of embryos for a logistic regression analysis with intercept and one covariate (e.g. t_{SB}) with $P < 0.05$ and power 0.8 and the levels of probabilities observed here is 274 (Hintze, 2013). Therefore, analysis with the logistic regression model shown in **Table 1** does not have the required power. The combined model of t_{SB} and age, however, has a significant effect of t_{SB} in the model (the AUC of the full model is lower than if the effect of t_{SB} was removed). From the logistic regression model, it can be seen that, for each year, the patient age increases, the odds ($1/(1-P)$ where P is the probability) of having an aneuploid embryo increases by 18% and that, for each hour, the value of t_{SB} increasing the odds of getting an aneuploid embryo increases by 5%.

For a specific patient in a specific treatment cycle, in which age is constant, we can expect the odds of aneuploidy to increase 5% per hour that t_{SB} increases. These results should however be treated with caution, since larger numbers are required to perform the logistic regression analysis. Future analysis with more data may confirm these findings. But it underlines again that both age and morphokinetics are important when determining the actual risk of aneuploidy. The aneuploidy risk classification model (Campbell et al., 2013a) was developed to evaluate the relative risk of aneuploidy for any embryo within a patient cohort. As the model is used to rank embryos for a specific patient, age does not change and should not be considered.

In conclusion, our position remains unchanged; indeed, it is strengthened by the statistical evaluation presented here. Although incidence of aneuploidy is increased with maternal age and age is correlated to the morphokinetic variables identified, this correlation is not strong. We do not consider the likely causal factor of observed periblastulation delays, to be maternal age and reject the challenges made by Ottolini et al. (2013) regarding this association. Our data clearly suggests that the delays are strongly associated with aneuploidy itself. We believe that this accessible and noninvasive embryo selection model may be used to classify the relative aneuploidy risk for embryos from a given patient electing against invasive genetic screening technology or for clinics without the skills or access to preimplantation genetic screening (PGS) (Campbell et al., 2013b). Alternatively, as Montag suggests (2013), the model could be used as an economic approach to prioritize low-risk blastocysts initially, for PGS. Euploid live birth data could enhance this model for prospective embryo selection and we believe that this should be the ultimate outcome measure. Again, we advise a cautious approach when considering transferring such morphokinetic embryo selection models between centres without prior validation.

We respectfully thank Ottolini, Rienzi and Capalbo for their interest in our recent articles and we encourage debate into this fascinating area of clinical embryology.

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Declaration: Statistical analyses were performed by Mette Laegdsmand PhD, Scientific Project Manager at Unisense Fertilitech, Denmark. Unisense Fertilitech is the manufacturer of the EmbryoScope, the time lapse device used in these studies.

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ARTICLE

Retrospective analysis of outcomes after IVF using an aneuploidy risk model derived from time-lapse imaging without PGS

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Alison Campbell studied at Leicester and Nottingham universities, specializing in assisted reproduction technology. Alison has held a senior embryology position with CARE Fertility since its inception in 1997. Since then she has played a key role in establishing new laboratories and has headed up the embryology teams across the organization. Alison's current role involves driving standards, best practice and leading research and development across the CARE laboratories in the UK and Ireland. Alison is an experienced clinical embryologist, a member of the HFEA-licensed centres panel and a diplomate of the Royal College of Pathologists. Alison was responsible for the first clinical application of time-lapse microscopy in the UK.

Abstract Time-lapse imaging of human preimplantation IVF embryos has enabled objective algorithms based on novel observations of development (morphokinetics) to be used for clinical selection of embryos. Embryo aneuploidy, a major cause of IVF failure, has been correlated with specific morphokinetic variables used previously to develop an aneuploidy risk classification model. The purpose of this study was to evaluate the effectiveness and potential impact of this model for unselected IVF patients without biopsy and preimplantation genetic screening (PGS). Embryo outcomes – no implantation, fetal heart beat (FHB) and live birth (LB) – of 88 transferred blastocysts were compared according to calculated aneuploidy risk classes (low, medium, high). A significant difference was seen for FHB ($P < 0.0001$) and LB ($P < 0.01$) rates between embryos classified as low and medium risk. Within the low-risk class, relative increases of 74% and 56%, compared with rates for all blastocysts, were observed for FHB and LB respectively. The area under the receiver operating characteristic curve was 0.75 for FHB and 0.74 for LB. This study demonstrates the clinical relevance of the aneuploidy risk classification model and introduces a novel, non-invasive method of embryo selection to yield higher implantation and live birth rates without PGS.

KEYWORDS: aneuploidy, blastocyst, embryology, IVF, live birth, time-lapse imaging

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Introduction

Since the advent of successful human IVF, fertilization and subsequent embryo culture has moved from the test-tube, held in sealed glass desiccators in 'warming cabinets', to the Petri dish in conventional culture incubators (Fishel and Edwards, 1982; Steptoe and Edwards, 1978), the latter being used clinically worldwide for more than three decades. Recently the introduction of time-lapse imaging had permitted the observation of zygotes and embryos as they pass through fertilization and syngamy to cleavage, compaction, blastulation and even eventual hatching. This novel technology permits the recording and retrospective analysis of temporal morphokinetic variables (Meseguer et al., 2011), not only in varying culture conditions but in a range of clinical scenarios. However, only one commercially available system, the EmbryoScope, provides for uninterrupted culture, for up to 6 days if required, therefore standardizing this variable. Cruz et al. (2011), following a safety evaluation of this time-lapse system, concluded that the periodic light exposure required for image acquisition in the EmbryoScope did not impair embryo quality or reproductive outcome. It has since been demonstrated that culturing in this system improves the incidence of clinical pregnancy (Meseguer et al., 2012). Also, very recently it was demonstrated using this system that aneuploid and euploid embryos have differing morphokinetic variables under standardized IVF culture conditions (Campbell et al., 2013).

The vital decision as to which of a patient's embryos should be preferentially selected for transfer following IVF treatment has to date been based primarily on a sequence of two to six chronological observations of the developing embryos. Standard IVF practice currently requires the removal of the culture dish containing the embryos from the incubator into suboptimal ambient conditions, in order for the embryologists to assess each embryo using light microscopy and record their observations relating to the number of cells, the stage of development and morphological features. This is typically performed once daily to minimize disturbance of the embryos in culture. For the vast majority of IVF clinical treatments worldwide, it is the scribed record of this series of static observations along with the status of the embryo at the time of the transfer procedure that dictates which embryo is selected. Various grading schemes exist and a consensus document for embryo grading was recently published in the hope of providing evidence-based guidelines and standard terminology for the accurate reporting of embryo development (Alpha and ESHRE, 2011a,b).

However, the viability of embryos is compromised by a significant incidence of aneuploidy, both meiotic (gametes) or mitotic (post fertilization) derived, which is a major cause of IVF failure and miscarriage (Fragouli and Wells, 2011; Kuliev et al., 2011). Furthermore, despite the ability to assess chromosome copy number (CCN), to date there has been no proven significant correlation between aneuploidy versus euploidy based on static morphological assessment of embryo preimplantation development for reliable clinical application (Fishel et al., 2010). To assess embryo ploidy with >95% accuracy, invasive biopsy of the embryo is required followed by expensive preimplantation genetic screening (PGS) using one of a range of molecular genetics-based technologies to acquire detailed knowledge of each individual CCN (Fishel et al., 2011; Johnson et al., 2010).

Recent work has demonstrated that aneuploidy screening as a mode of embryo selection in IVF can improve treatment outcome even for patients with the lowest risk of aneuploidy. A randomized pilot study reported a significant increase in clinical pregnancy rate in good-prognosis patients whose embryos were screened for aneuploidy compared with the control group whose embryos were selected for transfer based on morphology alone (Yang et al., 2012). The purpose of the current study was to evaluate the effectiveness and potential impact of a previously established, morphokinetic-based aneuploidy risk classification model (Campbell et al., 2013) for unselected non-PGS IVF patients through time-lapse imaging.

Materials and methods

Data for this research were obtained from the treatment of 69 couples attending an independent IVF clinic (CARE Fertility, Manchester, UK) from April 2011 to December 2012. All protocols complied with the UK regulation (Human Fertilisation and Embryology Act, 1990, 2008). The study did not require ethical or institutional review board approval, having been performed according to previously validated procedures. This was, necessarily, a retrospective cohort study utilizing time-lapse technology (EmbryoScope, Unisense FertiliTech, Denmark) for the recording of embryo developmental variables and treatment outcome data.

Patient criteria

All patients with a known outcome — fetal heart beat (FHB), live birth (LB) or failed implantation — following intracytoplasmic sperm injection (ICSI), EmbryoScope culture and blastocyst embryo transfer were included in this study. Those where the fate of the embryo could not be confirmed, such as a double-embryo transfer resulting in a singleton pregnancy, were excluded. Female age ranged from 25 to 47 years (mean ± SD 36·6 ± 5·1 years).

Ovarian stimulation

Pituitary suppression was performed with a gonadotrophin-releasing hormone agonist (Suprecur, 0.5 ml s.c. daily; Sanofi Aventis, UK) or antagonist (Cetrotide, 0.25 mg daily; Merck Serono, UK). Ovarian stimulation was achieved using human menopausal gonadotrophin (Menopur; Ferring, UK) and/or recombinant FSH (Gonal-F; Merck Serono), with doses ranging from 150 to 600 IU per day according to patient type and response.

Oocyte retrieval and embryology

The detailed methodology for oocyte collection, ICSI and embryo culture using the Embryoscope has been described previously (Campbell et al., 2013). Briefly, ultrasound-guided oocyte collection was performed under sedation and

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oocyte–cumulus complexes were prepared for ICSI between 2–4 h following harvesting. Following ICSI, oocytes were placed individually in microwells of the EmbryoSlide, loaded into the EmbryoScope and cultured until 5 or 6 days after oocyte collection and ICSI. The integrated microscope of the EmbryoScope was programmed to acquire images of each fertilized oocyte every 20 min through seven focal planes. These time-lapse images were used for the assessment of fertilization and embryo development up to the point of embryo transfer. The time of insemination by ICSI was programmed into the EmbryoScope when the slide was loaded as the time point midway through the ICSI procedure. The EmbryoViewer image analysis software was used to log and display the precise timing of developmental events as they were assessed by the embryologists studying the images. All times were recorded in hours post insemination by ICSI and the 'annotations' (detailed recordings of embryo development) were completed prior to embryo transfer. All data were, therefore, blind to outcome.

The two morphokinetic variables used in the aneuploidy risk classification model that was retrospectively applied to the blastocysts in this study were defined as: (i) *t*SB, the time from insemination to the start of blastulation, when the first sign of a blastocoele cavity forming was visible; and (ii) *t*B, the time from insemination to the formation of a 'full blastocyst', when the blastocoele cavity filled the embryo, the inner cell mass and trophectoderm tissues were distinguishable from each other and there was no more than 10% increase in the outer diameter of the zona pellucida. Embryos were selected for transfer according to a combination of standard morphological grading and clinic-defined novel morphokinetic exclusion criteria which are not correlated to *t*SB or *t*B. *t*SB and *t*B were not used as selection criteria but were studied retrospectively in relation to the outcome of the embryo transfer.

Outcome measures

Clinical pregnancy was defined by the presence of a gestational sac with FHB during weeks 6–8 of gestation. LB was confirmed by patient completion of a clinic delivery outcome form with birth details that, by regulation, are reported to the UK regulative body, the Human Fertilisation and Embryology Authority.

Embryos with known implantation outcome confirmed by LB, FHB or failed implantation (defined as negative pregnancy test) consisted of all single-blastocyst transfers or double-blastocyst transfers with either a negative pregnancy test or at least two fetal hearts. The data acquired relating to the implantation status of these embryos were defined as known implantation data (KID). A KID value of 1 was given when the outcome for an embryo was positive and 0 when the outcome for an embryo was negative, for FHB and for LB, which were analysed separately. Rates were calculated for comparison according to the following formula, KID positive/(KID positive + KID negative) × 100%. The KID rate was calculated for FHB and for LB.

Due to the time lag in receiving obstetric outcome information following FHB detection, the number of LB KID in this dataset is relatively low compared with the number of FHB KID. Reporting of live birth (positive LB KID) occurs, at the earliest, 10 months after embryo transfer (gestational period plus a delay in the clinic receiving outcome feedback). In order to give the most realistic value for live birth, only KID negatives where the time of transfer was 10 months earlier than the statistical analysis were included in the calculation of LB KID rate.

Aneuploidy risk classification model

The aneuploidy risk classification model variables (*t*SB and *t*B) were previously established on a data set where the ploidy status of each embryo was assessed by trophectoderm biopsy and molecular analysis of CCN by either array comparative genomic hybridization or single-nucleotide polymorphism array (**Table 1**; Campbell et al., 2013). This model was retrospectively applied to the KID data set of standard ICSI blastocyst-transfer cycles, without trophectoderm biopsy, in order to perform an independent validation of the potential benefit of the risk classification model. The model was used to retrospectively classify the risk of aneuploidy (low, medium or high) for each transferred embryo with a KID value (positive or negative).

Statistical analysis

The statistical software package R version 2.15.0 (The R Foundation for Statistical Computing) was used to calculate the means, variances and area under the receiver operating characteristic (ROC) curve. A Fisher's Exact test for count data was used to test if the classes had different incidences of positive and negative outcome.

Results

When the aneuploidy risk classification model was applied to the KID data set, the medium- and low-risk classes for aneuploidy were significantly different from each other with respect to known implantation rates for FHB ($P < 0.0001$) and LB ($P = 0.01$) (**Table 2**). Of the few embryos classified as high risk subsequent to transfer, none implanted. The aneuploidy risk classification model, therefore, indicated the predictive power for successful implantation and LB,

Table 1 The previously established three-class aneuploidy risk model based on trophectoderm biopsy data.

Risk class	*Definition*	
	tB (hpi)	*tSB (hpi)*
Low risk	<122.9	<96.2
Medium risk	<122.9	≥96.2
High risk	≥122.9	–

Source: Campbell et al. (2013).
hpi = hours post insemination; *t*SB = time from insemination to the start of blastulation; *t*B = time from insemination to the formation of a 'full blastocyst'.

Table 2 Known implantation data rates for fetal heart beat and live birth for each aneuploidy risk class.

Risk class	*FHB KID*		*LB KID*	
	No. of embryos	*FHB KID rate*	*No. of embryos*	*LB KID rate*
All	88	42.0	46	39.1
Low	33	72.7[a]	18	61.1[b]
Medium	51	25.5[a]	26	19.2[b]
High	4	0	2	0
Area under the ROC curve	0.75		0.74	

LB KID data were calculated only from treatments where the information could have been obtained (over 10 months from time of embryo transfer).
FHB = fetal heart beat; KID = known implantation data; LB = live birth.
[a] $P < 0.0001$.
[b] $P < 0.01$.

demonstrated by the area under the ROC curve being 0.75 for FHB and 0.74 for LB.

The incidence of positive FHB in the low-risk class was 72.7%, which represents a relative increase of 74% compared with the overall rate across all classes of embryos (42.0%). Correspondingly, the incidence of positive LB in the low-risk class was 61.1%, representing a relative increase of 56% when compared with the LB rate overall (39.1%).

To date, there has been one miscarriage reported following detection of a positive (single) FHB. The remaining FHB KID positives in this dataset were at least at 16 weeks of gestation. It is anticipated that the final relative increase in the low-risk class compared with overall LB rate will be higher than is currently reported, due to outstanding obstetric outcome data and based on the clinic's overall data (blastocyst transfer data 2009–2011: 11.6% miscarriage rate post-positive FHB, 730 LB positive and 826 FHB positive).

Individual known outcomes were plotted on charts depicting the temporal boundaries derived from the aneuploidy risk classification model according to their *t*SB and *t*B values (**Figures 1 and 2**).

Discussion

FHB and LB outcomes following the transfer of blastocysts, selected without the use of the PGS or the morphokinetic variables *t*SB and *t*B, were used to retrospectively evaluate the potential impact of a published aneuploidy risk classification model (Campbell et al., 2013). The current study demonstrates for the first time, as far as is known, that time-lapse imaging using defined morphokinetic data within a closed system for uninterrupted culture can be used to classify human preimplantation embryos according to their risk of aneuploidy, without performing biopsy and PGS, and that this correlates well with clinical outcome. The

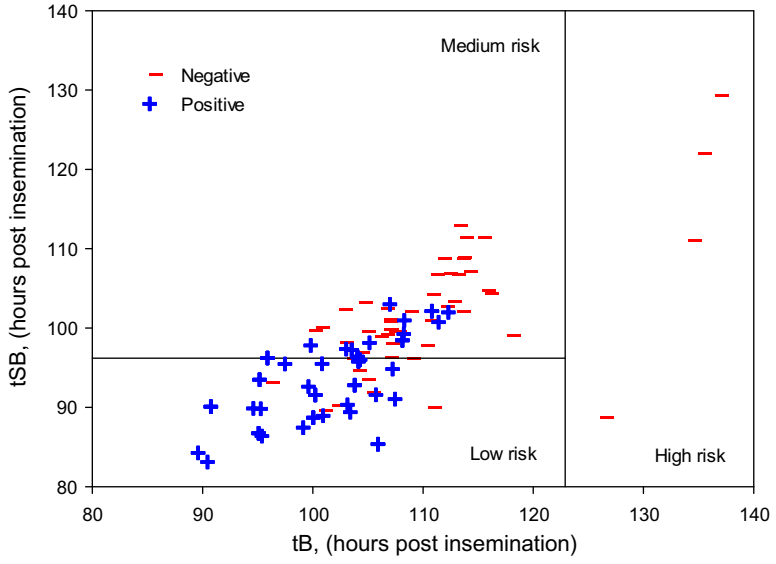

Figure 1 The previously established three-class aneuploidy risk model applied to embryos with known fetal heart beat outcome (gestational weeks 6–8). Blue crosses represent embryos that resulted in a fetal heart beat and red lines represent embryos that did not result in a fetal heart beat. Area under the ROC curve is 0.75. *t*SB = time from insemination to the start of blastulation; *t*B = time from insemination to the formation of a 'full blastocyst'.

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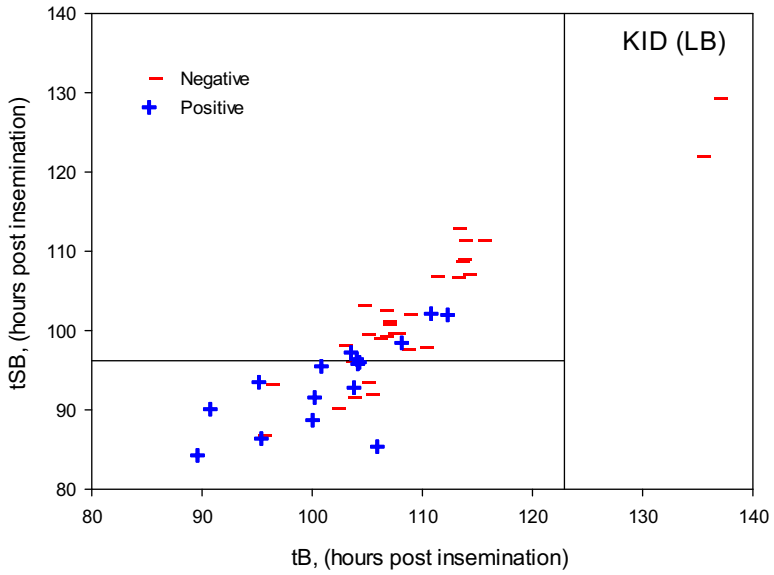

Figure 2 The previously established three-class aneuploidy risk model applied to embryos with known live birth outcome. Data were calculated only from treatments where the information could have been obtained (over 10 months from time of embryo transfer). Blue crosses represent live birth embryos and red lines represent embryos that did not result in a live birth. Area under the ROC curve is 0.74. *t*SB = time from insemination to the start of blastulation; *t*B = time from insemination to the formation of a 'full blastocyst'.

opportunity to identify and avoid selecting embryos at high risk of implantation failure using non-invasive objective criteria has been the main driver behind a welter of algorithms and static morphometric grading schemes for more than three decades (Fishel and Edwards, 1982; Fishel, 1986; Alpha and ESHRE, 2011a,b). The importance of linking this work to ploidy is that aneuploid embryos represent the largest single cause of failure and miscarriage following IVF.

Without time-lapse imaging and appropriate morphokinetic algorithms of the later stages of in-vitro embryo development, aneuploidy risk classification of blastocysts by any other non-invasive assessment has inadequate statistical power for clinical use. During the early post-fertilization period, there are mixed reports on the morphokinetic developmental criteria of embryos. Davies et al. (2012), using polar body biopsy for assessing CCN, described delayed early cleavage divisions of aneuploid compared with euploid embryos, and Chavez and colleagues (2012) reported a correlation between some early developmental parameters of 4-cell embryos and their CCN from individual blastomeres. The current study group using time-lapse imaging revealed that aneuploid and euploid embryos develop similarly at least until embryonic genome activation at around the 8-cell, day-3 stage, but during the peri-blastulation phase, aneuploid embryos have a significant delay in development compared with euploid embryos (Campbell et al., 2012).

CCN as defined by PGS technologies gives highly accurate data on embryo ploidy, although there is debate as to when the embryo biopsy should be performed for optimal clinical outcome. False positives and negatives are still apparent in some cases due to factors such as developmental arrest, mosaicism and putative repair mechanisms (Cater et al., 2012; Nasmyth and Haering, 2009). Determining embryo CCN is increasingly being considered as more reliable using a multicellular trophoblast biopsy at the blastocyst stage than a single blastomere of an 8-cell embryo on day 3, or inference of oocyte or zygote ploidy status from polar body biopsies. Aneuploidy is reported to be as high as 80% in early cleavage embryos of good morphology, deemed suitable for transfer, and even higher in those rarely chosen for transfer based on morphology (Jaroudi et al., 2012). The risk classification of aneuploidy in embryos is also most reliably performed at the peri-blastulation stage for IVF patients due to insignificant differences in a range of morphokinetic variables between the aneuploid or euploid status of early cleavage embryos (Campbell et al., 2013). Using conventional microscopy with static imaging, Alfarawati et al. (2011) demonstrated a weak association between blastocyst morphology and aneuploidy and reported an insignificant trend toward aneuploid embryos showing slower progression to the most advanced blastocyst stages. Their study also saw more pronounced delays in embryos with complex or multiple, compared with simple or single, aneuploidy.

Precise morphokinetic criteria using time-lapse imaging were required to develop the aneuploidy risk classification model as applied in this study, but additional variables identifiable from time-lapse imaging that correlate with embryo ploidy may yet be discovered. To date, cell evenness, multinucleation and cell-cycle length have all been assessed and compared using time-lapse imaging between aneuploid and euploid embryos screened at different stages of development, but only *t*SB and *t*B were used in a predictive algorithm to classify risk of aneuploidy effectively.

This group's examination of embryo development with time-lapse imaging from fertilisation through to blastocyst where ploidy was confirmed by trophectoderm biopsy, suggests that fragmentation is a dynamic process which continually changes and may be a completely random process. Fragmentation was observed equally in euploid and aneuploid

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embryos and incidence or patterning was not correlated to ploidy.

Due to intrinsic and extrinsic factors, it is likely that selection algorithms and models developed using time-lapse imaging systems may not be directly transferable between clinics or even between the wide spectrums of patient populations. Optimal morphokinetic variables may alter according to indication for IVF treatment, endocrinological profile or age (Leibenthron et al., 2012), for example, and differ according to gas tension or media used for embryo culture (Ciray et al., 2012; Kirkegaard et al., 2013). For these reasons, despite the likelihood that delays in aneuploid embryos, compared with euploid, will be seen during the periblastulation period generally, the values for *t*SB and *t*B used in the model that Campbell et al. (2013) developed and tested on independent embryo data may not be directly applicable and will need to be assessed in other settings. To ensure reliable data, it is also essential that strict adherence to standard policies (operating procedures) is required. However, as has been demonstrated, locally defined conditions with strict adherence to protocol can provide predictive algorithms which promise an improved incidence of live birth following IVF.

Larger data are required to test whether the aneuploidy risk classification model is more effective for particular patients or embryos than others and whether particular and specific numerical chromosomes have differing effects on embryo development, aneuploidy risk classification and fate. Miscarriage rates were considered within each risk classification and, within this dataset, there was one miscarriage reported following FHB confirmation. This was from the transfer of a blastocyst classified as medium-risk aneuploidy. Larger studies assessing miscarriage incidence within these aneuploidy risk classes would be of clinical interest.

This risk classification of aneuploidy in blastocysts could be used in conjunction with PGS to prioritize embryos for screening or as an alternative or routine selection method for enhancing live birth outcome where PGS is unavailable, either because it is not permitted due to local regulation or where a non-invasive approach is desired. Aneuploidy screening during IVF treatment is most commonly offered to a small group of patients considered to be at highest risk, usually with advanced female age, but with the increasing availability of safe and non-invasive time-lapse technology, a balanced view should be taken by patients and practitioners as to the most appropriate, effective and efficient method of embryo screening for them. Whilst the technology for genetic screening is widely available, patient access to it may also be limited by the lack of technical expertise in IVF laboratories, the low proportion of IVF centres that have established PGS programmes and its high cost. The possibility of being able to classify the risk of aneuploidy in an embryo using non-invasive imaging only provides any clinic and their patients an accessible lower cost alternative which avoids additional handling and biopsy of the embryo during its preimplantation development. Embryo selection based predominantly on specific time-lapse derived algorithms could rapidly become routine in IVF treatment. This study group believes that using this non-invasive technology to screen out embryos with the highest risk of aneuploidy — a major cause of IVF failure — will result in a paradigm shift in the clinical practice of human conception *in vitro* and improve the incidence of live birth for most patients.

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4.0 Specific aim 3.

To collaborate to perform a multicentre outcome analysis to consider the limitations of a time-lapse blastocyst prediction model.

Encapsulated in this specific aim is the article demonstrating the limitations of a blastocyst prediction model and discussion of the advantages of developing time lapse algorithms predictive of live birth potential.

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In this study, my personal contribution was preparation of a large proportion of the data included in the analysis, interpretation of statistical results, writing and manuscript preparation.

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COMMENTARY

Limitations of a time-lapse blastocyst prediction model: a large multicentre outcome analysis

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Abstract The goal of embryo selection models is to select embryos with the highest reproductive potential, whilst minimizing the rejection of viable embryos. Ultimately, any embryo selection model must be tested on clinical outcome. We therefore retrospectively tested a published blastocyst prediction model on a large combined set of transferred embryos with known clinical outcome. The model was somewhat effective in that we found a relative increase of 30% for implantation in the model-selected group of embryos. There was, however, a concomitant large rejection of embryos from our test cohort, which actually resulted in pregnancy. This hypothetical experiment highlights the limitations of predicting blastulation only. Crucially, it illustrates that both sensitivity and specificity are important parameters when developing embryo selection models for prospective clinical use.

KEYWORDS: assisted reproduction, embryo selection, prediction model, time lapse

Introduction

Time-lapse imaging of human preimplantation embryos has become rapidly integrated in IVF laboratories. The proposed advantages, such as uninterrupted embryo culture, flexibility in timing, improvement of documentation procedures, quality control and management and, in particular, the introduction of dynamic markers of embryo quality, have altogether stimulated a profound interest in time-lapse technology. While a large number of publications consolidate that timing of development differs between viable and nonviable embryos (Herrero and Meseguer, 2013), only a few publications offer clinically applicable models of embryo selection (Campbell et al., 2013; Conaghan et al., 2013; Meseguer et al., 2011). Yet, as recently demonstrated, a proposed multivariate hierarchical selection model was not transferable from one clinical setting to another without modification (Best et al., 2013). It has been speculated that a less-complex model, such as the one recently developed and applied by Conaghan et al. (2013) that categorized embryos

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into groups with either high or low likelihood of forming 'usable blastocysts', could be applicable to other clinics. The model has, however, not been evaluated with regard to clinical outcome. To test this hypothesis and correlation between the published time intervals and clinical outcome, we retrospectively applied the same model to a large set of transferred embryos from independent clinics.

Retrospective testing of a blastocyst prediction model

Seven clinics from three different countries participated by contributing data on clinical outcome following embryo transfer (fetal heart beat) and timing of cellular divisions until day 3, obtained using time-lapse monitoring (EmbryoScope, FertiliTech, Denmark). The first division was annotated t_2, second division t_3 and the third division t_4.

A total of 1519 transferred embryos with known outcome for implantation from cycles with single ($n = 517$) or double ($n = 501$) embryo transfer were included. In order to be able to relate each embryo's fate after transfer with its individual morphokinetic profile, only cycles with two or no fetal heart beats were included where double-embryo transfers were performed. This implies that the presented pregnancy rates are lower and not directly comparable with treatment success rates. Patient stimulation, IVF/intracytoplasmic sperm injection and embryo culture was performed according to standard procedures at each site. Embryos were graded and selected according to each clinic's routine methodology and one or two embryos were selected for transfer. Embryo transfer was predominantly performed on day 2 or day 3.

In order to test whether time intervals published in Conaghan et al. (2013) correlated with clinical outcome, we retrospectively grouped the transferred embryos into usable and nonusable embryos based on the model's values for these time-lapse intervals. The model was found to predict a high chance of usable blastocyst formation (defined as a blastocyst suitable for either transfer or freezing) if time between first and second cytokinesis ($t_3 - t_2$) was 9.33–11.45 h and time between second and third cytokinesis ($t_4 - t_3$) was 0–1.73 h. Likewise, embryos were predicted to have a low chance of forming usable blastocysts if $t_3 - t_2$ and $t_4 - t_3$ were longer than these time intervals. We calculated the relative difference in implantation (%) between the usable group and the entire cohort, odds ratio for implantation in the usable compared with the nonusable group and the percentage of nonusable embryos that resulted in implantation. Data were used to generate a receiver operating characteristic curve and to calculate area under the curve for implantation.

Data for implantation are presented in **Table 1**. The relative difference in implantation rate between the entire cohort and the embryos categorized as usable by the test model was 30.0%. The odds ratio for implantation between usable and nonusable was 1.60. The sensitivity was 0.50 and the specificity was 0.65. Furthermore, 50.6% of the embryos that resulted in pregnancy were categorized as nonusable according to the model. The area under the curve for prediction of implantation was 0.57 (**Figure 1**).

In other words, applying the test model retrospectively to transferred embryos from the independent clinics would have provided an increase of 30.0% in implantation rate for embryos grouped as usable compared with the entire test cohort. Notably, out of the embryos that actually implanted, 50.6% were categorized as having low chance of being usable. This indicates that relying on such a model would bring a substantial risk of deeming viable embryos nonusable.

Table 1 Implantation data for embryos categorized as usable or nonusable according to the test model.

	Implanted	*Not implanted*	*Implantation rate*
Usable	131	445	22.7
Nonusable	134	809	14.2
Entire cohort	265	1254	17.4

Values are *n* or %.
Usable: $t_3 - t_2 = 9.33$–11.45 h and $t_4 - t_3 = 0$–1.73 h. Unusable: $t_3 - t_2$ outside 9.33–11.45 h and $t_4 - t_3$ outside 0–1.73 h.

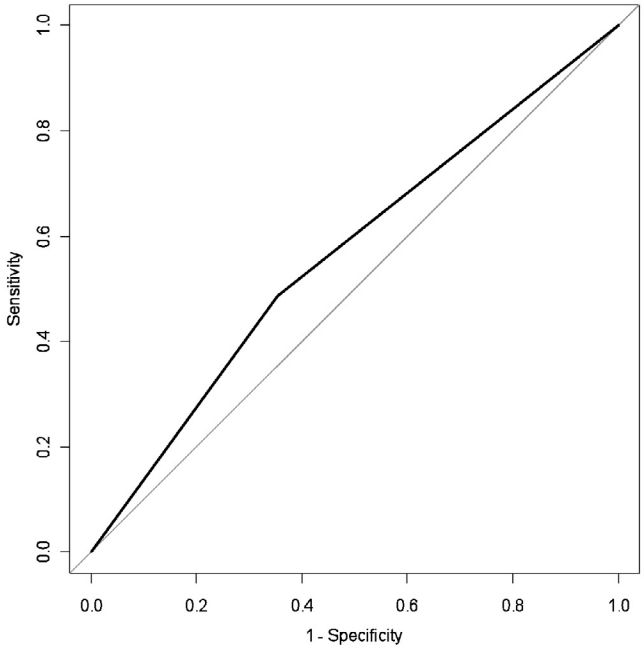

Figure 1 Receiver operating characteristic curve for prediction of pregnancy by the parameters $t_3 - t_2$ and $t_4 - t_3$ from the test model.

The ultimate goal of embryo selection models is to positively select embryos with the highest reproductive potential, notably without rejecting viable embryos. The premise of this hypothetical experiment is that the test model predicts the formation of usable blastocysts i.e. blastocysts to be either transferred or frozen. If embryos are selected for transfer/freezing on day 3 (as suggested by the authors), it ultimately follows that blastocysts with a low chance of being usable are to be discarded if the model is applied uncritically. Principally, embryos from this study population that have implanted would have been discarded with day-3 transfer and application of the time-lapse-based selection model.

This hypothetical experiment illustrates the risks of defining too narrow time intervals for optimal division in order to achieve a high specificity at the expense of a low

sensitivity. It thus underlines the importance of carefully considering that a model must not only provide a substantial increase in implantation but also, equally important, that a low rejection rate of viable embryos is secured. This very important point is demonstrated by applying the time-lapse criteria on an unprecedented large set of transferred embryos with a known outcome.

From blastocyst to pregnancy and beyond

The test model was developed in order to identify viable embryos from a cohort, while the study population is constituted by embryos selected for transfer with clinical outcome as the endpoint. Thus, both the endpoint and the study population differ between the two studies. It would be expected that a model that predicts blastocyst development would be different from a model that predicts clinical outcome. In our opinion, it would, however, be expected that a model that predicts blastocyst development would positively select more embryos than a selection model predictive of implantation and clinical pregnancy, as embryos resulting in pregnancy would constitute a subgroup of embryos that develop into blastocysts. We do not, however, find any explanation, neither in the different study populations nor in the different endpoints, as to why a large proportion of embryos which were rendered 'unusable' by the model as they fell outside the model selection criteria resulted in implantation. In our opinion, the most likely explanation is the narrow time intervals for optimal cellular division.

Ultimately, any embryo selection model must be tested on clinical outcome, as blastocyst development is a surrogate endpoint, preferably in a prospective study. We believe that the approach of retrospectively testing the criteria on transferred embryos with known outcome is justified in this case, as it aids the design of future prospective studies.

Our study supports an approach where models are developed with appropriate concern for low rejection of viable embryos, where clinical outcome is used as an endpoint and where the model is individually adjusted to specific settings and validated prior to implementation.

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5.0 Specific aim 4.

To amass time lapse data during clinical IVF treatments to make novel observations and to develop in house embryo selection algorithms to predict implantation and live birth and to compare the efficacy of such algorithms with conventional morphological selection.

Encapsulated in this specific aim are the studies below describing the evolution of time lapse algorithm development and the identification of novel variables.

*Fishel S, **Campbell A**, Montgomery S, Smith R, Nice L, Duffy S, Jenner L, Berrisford K, Kellam L, Smith R, D'Cruz I, Beccles A. Live births after embryo selection using morphokinetics versus conventional morphology: a retrospective analysis. Reprod Biomed Online. 2017 Oct;35(4):407-416. doi: 10.1016/j.rbmo.2017.06.009. Epub 2017 Jun 23. PubMed PMID: 28712646.*

*Fishel S, **Campbell A**, Montgomery S, Smith R, Nice L, Duffy S, Jenner L, Berrisford K, Kellam L, Smith R, Foad F, Beccles A. Time-lapse imaging algorithms rank human preimplantation embryos according to the probability of live birth. Reprod Biomed Online. 2018 Sep;37(3):304-313. doi: 10.1016/j.rbmo.2018.05.016. Epub 2018 Jun 22. PubMed PMID: 30314885.*

Fishel S, ***Campbell A****, Foad F, Davies L, Best L, Davis N, Smith R, Duffy S, Wheat S, Montgomery S, Wachter A. Evolution of embryo selection for IVF from subjective morphology assessment to objective time lapse algorithms improves chances of live birth. Reprod Biomed Online. 2019 in press*

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Here, my personal contributions were:

- To present the results of several small studies in the form of abstract presentations at national and international conferences to raise awareness of time lapse technology and the benefits for patients and scientists. (See appendix 2 for a selection of presented poster abstracts)
- Data compilation and preparation for algorithm development.
- Scoping of the analyses required.
- Design of the studies.
- Interpretation of statistical analyses.
- Manuscript preparation: co-writing of the first draft and amendments following review.
- Corresponding author on two of these publications.

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Article

Live births after embryo selection using morphokinetics versus conventional morphology: a retrospective analysis

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Simon Fishel, Founder and President of the CARE Fertility Group, worked with IVF pioneer and Nobel Laureate Robert Edwards from 1975 to 1985 at Cambridge University and as Deputy Scientific Director of the first IVF clinic, Bourn Hall, from 1980. In 1978 he received the prestigious Beit Memorial Fellowship and was elected a Research Fellow of Churchill College, Cambridge, publishing over 200 papers and four books. He was the first to publish on the adaptation of the mammalian to its environment, and in 1984 on the secretion of hCG by the human embryo. In 1992 he founded the world's first degree course in IVF and in 2009 was honoured by the Liverpool John Moores University with their highest award of 'University Fellow' for "*outstanding contribution to science and to humanity*".

KEY MESSAGE
Incidence of live birth after embryo transfer using morphokinetic algorithms during uninterrupted culture to select embryos was increased by 19% compared with conventional morphology and standard incubation in women younger than 38 years. Incidence of aneuploidy may limit their effectiveness in women older than 37 years.

A B S T R A C T

The increasing corpus of clinical studies using time-lapse imaging for embryo selection demonstrates considerable variation in study protocols and only limited-sized study cohorts. Outcome measures are based on implantation or clinical pregnancy; some predict blastulation from early cleavage-stage data, and few have evaluated live birth. Erroneously, most studies treat the embryos as independent variables and do not include patient or treatment variables in the statistical analyses. In this study, cohort size was 14,793 patients and 23,762 cycles. The incidence of live birth (n = 973 deliveries) after embryo selection by objective morphokinetic algorithms was compared with conventional embryology selection parameters (n = 6948 deliveries). A 19% increase in the incidence of live birth was observed when morphokinetic data were used to select embryos for the patient cohort aged younger than 38 years (OR 1.19 with 95% CI 1.06 to 1.34) using their own eggs, and an increase of 37% for oocyte recipients aged over 37 years (OR 1.370; 95% CI 0.763 to 2.450). This is the largest study of the prospective use of time-lapse imaging algorithms in IVF reporting on live birth outcome, although the nature of purely a closed system versus standard incubation could not be assessed.

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Introduction

The success of IVF during the last 35 years has evolved from aspirational live birth rates of around 15% (Edwards et al., 1984; Fishel et al., 1984, 1985) to rates of between 35 and 60% (HFEA, 2013). This is largely dependent on female age, and is a result of advances in follicular stimulation regimens, multiple embryo transfer and improvements in the culture and selection of the human embryo for transfer. In recent years, with the more responsible adoption of single- or double-embryo transfer after IVF, permitted by further improvements in culture media, the need for embryo-selection tools has been ever more pressing to ensure high live birth rates. Human conception is inefficient, with estimates of up to 70% of human zygotes being non-viable in their progression to a healthy full-term delivery (Zinaman et al., 1996). The first in-vitro evidence for aneuploidy as a potential cause of human embryo failure was published in the early 1980s (Angell et al., 1983, 1986). Irrespective of chromosome copy number, since the origins of IVF, human morphokinetics was enormously important in predicting viable embryos (De Neubourg et al., 2004; Edwards et al., 1984; Fishel et al., 1983). For many clinics, the goal is single embryo transfer while still improving the incidence of healthy live birth outcomes to levels unimaginable only 2 decades ago, thereby minimizing the unacceptable risk of high-order multiple pregnancies. For the highest chance of success per cycle, however, IVF practice involves ovarian stimulation usually resulting in several embryos, from which one, or at most two, are selected for transfer. Confidence in the elective transfer of a single embryo has been achieved, in part, by advances in embryo culture and technology. The development of time-lapse imaging (TLI) in recent years (Lemmen et al., 2008; Meseguer et al., 2011) has enabled better embryology laboratory practice in several domains: workflow, consistent objective selection, and zygote and embryo protection as part of the putative improved culture conditions using continuous, undisturbed culture system.

The evidence for using sophisticated time-lapse systems for morphokinetic algorithms that are predictive of successful outcome, however, is limited (Kaser and Racowsky, 2014). In this retrospective analysis of treatments undertaken from January 2010 to January 2015, live birth outcome of a large cohort using our time lapse imaging protocols as a prospective selection tool for embryo transfer was compared with those patients undergoing conventional embryology. The time-lapse system used closed incubation (EmbryoScope), and generated the algorithms from morphokinetic data for the embryo selection, compared with standard incubation and the use of conventional morphological embryo selection according to The Istanbul Consensus (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011).

Materials and methods

This multicentre study included 14,793 patients from six CARE Fertility centres from January 2010 to January 2015: CARE Nottingham, CARE Northampton, CARE Manchester, CARE Sheffield, Beacon CARE Fertility Dublin and CARE London; representing a total of 21,235 treatment cycles in the standard treatment group and 2527 in the EmbryoScope treatment group, with 6948 (32.7%) and 973 (38.5%) live births observed, respectively . 'Patients' or 'non-recipients' (n = 21,466 cycles) comprised those who used all their own oocytes (n = 20,664) and patients who were also oocyte-share donors (n = 802); 'recipients' comprised those women undergoing oocyte donation (n = 2296 cycles). All protocols for patient treatments complied with UK regulation (Human Fertilisation and Embryology Act, 1990, 2008) and all UK facilities are regularly inspected by the Human Fertilisation and Embryology Authority (HFEA). The retrospective analysis, or the use of time-lapse imaging algorithms for embryo selection, did not require ethical or institutional review board approval, as it was carried out according to previously validated procedures, and practised under license from the HFEA. All patients were counselled and gave signed consent. Time lapse imaging (TLI) was undertaken using the EmbryoScope (Vitrolife, Sweden) with strict adherence to annotation protocols, and all embryos were selected for transfer based on their in-house derived TLI algorithm score for each stage of transfer; patient choice dictated whether they used EmbryoScope associated with morphokinetic algorithm for embryo selection or standard incubation. The primary end-point of this study was live birth events; that is, the number of patients achieving a delivery of a live born infant. Only 'fresh' embryo transfer cases were included, and all pre-genetic implantation screening cases were excluded.

The following clinical variables were categorized for inclusion in the analysis: patient age, day of embryo transfer, number of embryos transferred, donor age (where applicable), body mass index (BMI), anti-Müllerian hormone (AMH), antral follicle count (AFC), gonadotrophin type, gonadotrophin dosing days, and gonadotrophin total dose. The groupings applied are presented for each variable in **Table 1**. Patient age was considered as a binary variable in the modelling (younger than 38 years and 38 years and over), corresponding to the common grouping used by HFEA, which is familiar to patients. The groupings for categorizing BMI, AMH, and AFC were chosen to correspond to clinically meaningful categories, i.e., to reflect what might be considered to be above, below or within a normal or healthy range. The donor ages (less than 29 years, 29–32 years and 33 years and over), gonadotrophin number of days of dosing and the total dose variables were categorized on the basis of quantiles of the observed distribution to ensure that sufficient information was present in each of the categories for a robust analysis.

The following definitions were used for the BMI, AMH and AFC categories, relating to the data presented: BMI less than 18.5 (underweight), 18.5 and less than 25 (healthy weight), 25 and less than 30 (overweight), 30 and less than 40 (obese), and 40 upwards (extremely obese); AMH (pmol/L): less than 6 (low), 6 and less than 24 (normal), 24 and less than 70 (high), 70 upwards (very high); AFC (number of follicles seen on ultrasound scan): less than 4 (extremely low), 4 and less than 10 (low), 10 and less than 14 (somewhat low), 14 and less than 22 (normal), 22 and less tha 35 (high), 35 upwards (very high).

Ovarian stimulation protocols

Pituitary suppression for ovarian stimulation was carried out either with gonadotrophin-releasing hormone agonist (Suprecur; 0.5 ml subcutaneously daily; Sanofi Aventis, UK) or antagonist (Cetrotide; 0.25 mg daily; Merck Serono, UK). Ovarian stimulation was achieved using human menopausal gonadotrophin (Menopur, Ferring, UK), recombinant FSH (Gonal-F; Merck Serono), or both (as previously described (Campbell et al., 2013b; Fishel et al., 2016).

Table 1 – Clinical data available for the cycles in each treatment arm (EmbryoScope/standard).

Variable	Group/summary Statistic	Standard (*n* = 21235)	Embryoscope (*n* = 2527)	Total (*n* = 23762)
Patient age, years	<38	13447 (63.3)	1414 (56.0)	14861 (62.5)
	38 +	7788 (36.7)	1113 (44.0)	8901 (37.5)
Day of embryo transfer	2	4150 (19.5)	234 (9.3)	4384 (18.4)
	3	8790 (41.4)	924 (36.6)	9714 (40.9)
	4	419 (2.0)	146 (5.8)	565 (2.4)
	5 or 6	7876 (37.1)	1223 (48.4)	9099 (38.3)
Number of embryos transferred	1	10118 (47.6)	1057 (41.8)	11175 (47.0)
	2	10463 (49.3)	1470 (58.2)	11933 (50.2)
	3	654 (3.1)	0 (0.0)	654 (2.8)
Patient type	Oocyte share donor	774 (3.6)	28 (1.1)	802 (3.4)
	Donor recipient	1810 (8.5)	486 (19.2)	2296 (9.7)
	Standard	18651 (87.8)	2013 (79.7)	20664 (87.0)
Donor age, years	<29	585 (2.8)	182 (7.2)	767 (3.2)
	29–32	649 (3.1)	165 (6.5)	814 (3.4)
	33 +	576 (2.7)	139 (5.5)	715 (3.0)
	Not applicable	19425 (91.5)	2041 (80.8)	21466 (90.3)
ICSI	No	10515 (49.5)	763 (30.2)	11278 (47.5)
	Yes	10720 (50.5)	1764 (69.8)	12484 (52.5)
Intralipid	No	19364 (91.2)	2214 (87.6)	21578 (90.8)
	Yes	1871 (8.8)	313 (12.4)	2184 (9.2)
BMI[a], kg m^2	Underweight	167 (0.8)	24 (0.9)	191 (0.8)
	Healthy	6933 (32.6)	799 (31.6)	7732 (32.5)
	Overweight	3952 (18.6)	355 (14.0)	4307 (18.1)
	Obese	1322 (6.2)	152 (6.0)	1474 (6.2)
	Extremely obese	30 (0.1)	2 (0.1)	32 (0.1)
	Unavailable	8831 (41.6)	1195 (47.3)	10026 (42.2)
AMH, pmol/L[a]	Low	1071 (5.0)	139 (5.5)	1210 (5.1)
	Normal	1900 (8.9)	264 (10.4)	2164 (9.1)
	High	790 (3.7)	109 (4.3)	899 (3.8)
	Very high	57 (0.3)	6 (0.2)	63 (0.3)
	Unavailable	17417 (82.0)	2009 (79.5)	19426 (81.8)
AFC number of follicles seen on ultrasound scan[a]	Extremely low	484 (2.3)	59 (2.3)	543 (2.3)
	Low	1797 (8.5)	238 (9.4)	2035 (8.6)
	Somewhat low	1667 (7.9)	185 (7.3)	1852 (7.8)
	Normal	2539 (12.0)	213 (8.4)	2752 (11.6)
	High	1978 (9.3)	174 (6.9)	2152 (9.1)
	Very high	1111 (5.2)	67 (2.7)	1178 (5.0)
	Unavailable	11659 (54.9)	1591 (63.0)	13250 (55.8)
Gonadotrophin type	Other	353 (1.7)	63 (2.5)	416 (1.8)
	Gonal-F	2994 (14.1)	482 (19.1)	3476 (14.6)
	Menopur	9929 (46.8)	1452 (57.5)	11381 (47.9)
	Unavailable	7959 (37.5)	530 (21.0)	8489 (35.7)
Gonadotrophin dosing days	<10	2178 (10.3)	412 (16.3)	2590 (10.9)
	10–11	3156 (14.9)	569 (22.5)	3725 (15.7)
	12 +	8227 (38.7)	1035 (41.0)	9262 (39.0)
	Unavailable	7674 (36.1)	511 (20.2)	8185 (34.4)
Gonadotrophin total dose, iuiu iu	<1800	1973 (9.3)	420 (16.6)	2393 (10.1)
	1800–2699	3929 (18.5)	576 (22.8)	4505 (19.0)
	2700–3599	3822 (18.0)	464 (18.4)	4286 (18.0)
	3600 +	3442 (16.2)	532 (21.1)	3974 (16.7)
	Unavailable	8069 (38.0)	535 (21.2)	8604 (36.2)
Total previous cycles	Mean (SD)	2.4 (1.82)	2.5 (1.78)	2.4 (1.81)
	Median (range)	2.0 (0–25)	2.0 (0–18)	2.0 (0–25)
Total numbr of previous live births	Mean (SD)	0.3 (0.63)	0.3 (0.58)	0.3 (0.62)
	Median (range)	0.0 (0–9)	0.0 (0–6)	0.0 (0–9)
Total number miscarriages	Mean (SD)	0.4 (0.88)	0.5 (0.89)	0.4 (0.88)
	Median (range)	0.0 (0–15)	0.0 (0–7)	0.0 (0–15)
Duration of infertility	Mean (SD)	3.6 (2.85)	3.4 (2.63)	3.6 (2.83)
	Median (range)	3.0 (0–31)	3.0 (0–25)	3.0 (0–31)
Total number of ectopics	Mean (SD)	0.1 (0.35)	0.1 (0.28)	0.1 (0.34)
	Median (range)	0.0 (0–5)	0.0 (0–3)	0.0 (0–5)
Number of eggs collected	Mean (SD)	9.6 (4.98)	10.1 (5.20)	9.7 (5.02)
	Median (range)	9.0 (1–38)	9.0 (1–41)	9.0 (1–41)

(*continued on next page*)

Table 1 – (*continued*)

Variable	Group/summary Statistic	Standard (*n* = 21235)	Embryoscope (*n* = 2527)	Total (*n* = 23762)
Number of eggs inseminated	Mean (SD)	7.9 (4.34)	8.6 (4.71)	8.0 (4.40)
	Median (range)	7.0 (1–35)	8.0 (1–41)	7.0 (1–41)
Ratio of mature (metaphase 2) to total eggs collecte	Mean (SD)	0.3 (0.54)	0.2 (0.42)	0.3 (0.53)
	Median (range)	0.1 (0–12)	0.1 (0–7)	0.1 (0–12)
Number of eggs fertilized	Mean (SD)	5.2 (3.27)	5.9 (3.60)	5.3 (3.33)
	Median (range)	5.0 (0–26)	5.0 (1–30)	5.0 (0–30)
Patient age	Mean (SD)	35.5 (5.17)	36.7 (4.80)	35.6 (5.15)
	Median (range)	36.0 (19–54)	37.0 (22–50)	36.0 (19–54)
Recipient patient age	Mean (SD)	40.7 (4.92)	41.4 (4.40)	40.8 (4.83)
	Median (range)	41.0 (21–50)	42.0 (24–50)	42.0 (21–50)
Donor age (where applicable)	Mean (SD)	30.1 (3.91)	29.5 (4.05)	29.9 (3.94)
	Median (range)	31.0 (18–45)	30.0 (19–42)	30.0 (18–45)

[a] Cross-tabulations, showing the number (and percentage) of observations in each group, are provided for the binary or categorical variables considered in the analysis versus treatment. Summary statistics, mean (and SD) and median (and range), are provided for each count variable by treatment. Continuous summaries of patient age, donor age (where applicable) and patient age by donor status are also provided for completeness. [antr]For definition so fhte categories see Materials and Methods.

AFC, antral follicle count; AMH, anti-Müllerian hormone; BMI, body mass index; ICSI, intracytoplasmic sperm injection.

Oocyte retrieval, denudation and intracytoplasmic sperm injection

Female sedation was achieved with a combination of propofol (Braun, Germany), fentanyl (Auden McKenzie, UK) and midazolam (Hamelyn, UK), and transvaginal ultrasound-guided oocyte retrieval took place about 36 h after HCG injection (10,000 IU; Pregnyl; Organon, UK; or Ovitrelle; Merck Serono) or agonist trigger (Buserelin 0.5 ml sc; Suprefact, Sanofi S.A, France), using an aspiration needle (Vitrolife, Sweden) connected to a vacuum pump (Rocket Medical, UK). Oocyte–cumulus complexes were recovered from follicular aspirates using a stereomicroscope in a class II hood with a heated stage, washed and cultured in Ferticult IVF medium (Fertipro, Belgium) at 5% CO_2 in air, 37.0 ^{0}C, maximum humidity, Oocytes allocated for ICSI were cultured for 2–4 h before cumulus cell denudation with 15–20 IU/ml cumulase (Origio, Denmark) in the same medium and complete removal of the coronae radiatae with a 140 µm pipette (EZ Squeeze; Research Instruments, UK). Oocytes at the metaphase-II stage underwent insemination by intracytoplasmic sperm injection (ICSI) within 2 h of denudation. Oocytes allocated for IVF were inseminated after preparation using SupraSperm density gradient (Origio, Denmark) and washing in Ferticult IVF medium (Fertipro, Belgium) at a concentration of 0.2 M/ml, between 3 and 6 h after oocyte recovery and cultures in standard incubators for 18 + /– 1 h before fertilization was assessed.

Embryo culture and incubation

For time-lapse imaging, after ICSI or IVF, oocytes or zygotes, respectively, were placed individually in microwells of EmbryoSlides™ (Vitrolife, Sweden)) in 25 µl Global IVF medium (LifeGlobal) supplemented with 10% dextran serum supplement (Irvine Scientific) and were overlaid with 1.4 ml mineral oil (Fertipro, Belgium) and placed in the EmbryoScope. EmbryoSlides were prepared with medium and oil that had equilibrated overnight. Once loaded with the ICSI-inseminated oocytes, or zygotes after IVF, EmbryoSlides were placed into the EmbryoScope time-lapse incubator at 37.0^{0}C in 5.5–6.0% CO_2, 5% O_2 and 89.5% N_2 for up to 6 days. The built-in microscope was used to acquire images of each fertilized oocyte every 10–20 min through seven focal planes.

For standard incubation, fertilization and embryo culture was carried out in small volume box, or flatbed incubators (Galaxy 48R, New Brunswick, UK; Miri, ESCO, Japan), using the same media, mineral oil as described for the EmbryoScope (Vitrolife (Sweden) CE marked plasticware and 25 µl culture drops). As this was a multicentre study, up to 2012, less than 25% of standard incubation was atmospheric O_2 with an increasing switch to 5% O_2, resulting in the last 18 months of the study being 100% low O_2. Analysis of the data showed no significant effect of atmospheric O_2 on the analyses (data not shown). During the early phase of this work, the culture medium was refreshed on day 3 but after CARE pilot studies, this step was no longer used. All embryos were cultured singly in culture drops, irrespective of incubation method. Selection of the embryos from standard incubation was based primarily on morphological criteria (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011).

Evaluation of time-lapse images

Time-lapse images were collected for the duration of the culture period, to the point of embryo transfer, and were used for the assessment of fertilization after ICSI and during all in-vitro embryo development. The time of insemination by ICSI was programmed into the EmbryoScope when the slide was loaded, as the time point midway through the ICSI procedure. For IVF, the time of insemination was recorded as the time sperm was added to the oocytes. The EmbryoViewer® image analysis software (Vitrolife) was used to log and display the precise timing of developmental events as they were annotated by the embryologists studying the time-lapse images. The definitions of morphokinetic variables has been described in detail previously (Campbell et al., 2013a, 2013b). For the present study, the early cleavage model avoided the selection of embryos where irregular or rapid division occurred cc2 < 2h and cc2>/=2 h or cc3<5 h and cc3>/=5 h and where t2>/=27.1 h and t2 <27.1 h. All times were after ICSI insemination, and a 1.95 h adjustment was made for IVF (based on our in-house data, unpublished data). The duration of cc2 (t3–t2) relative to cc2+cc3 (t5–t3) {relcc2} was an effective splitting criterion if the aforementioned irregular or rapid divisions were not present. The optimum value for relCC2 was more than 0.44 and less than 0.47.

Embryos with multinucleation at the four-cell stage or smooth endoplasmic reticulum clusters were also avoided. For blastocysts, these were preferentially selected with a tSB (time to the start of blastulation in hours after insemination) ≤ 93.1. If this criterion was not met, preference was given to embryos where dB (tB–tSB) ≤ 12.5 h.

All times were recorded in hours after insemination. All annotations were made before any decision on embryo transfer, with the annotation criteria outlined above being the arbiter of which embryo to transfer.

Embryology annotation protocols and quality control

After training in annotation and competency assessment, CARE embryologists participate in regular quality assurance exercises and use a centralized annotation quality-assurance protocol whereby example embryos are annotated by each practitioner and their values compared with those of their colleagues. Intra-correlation coefficients are calculated for each morphokinetic value. Annotation quality is considered assured where the intra-correlation coefficients are greater than 0.9, demonstrating close correlation between practitioners, and competent annotation.

Embryo selection

After TLI, embryos were objectively selected using user defined time-lapse algorithms programmed into the 'Compare and Select' software (EmbryoViewer®, Vitrolife, Sweden – Class 1 Medical Device). Model scores were used to rank embryos according to their implantation or live birth potential. An evolving series of models were used during this study period as they were fine-tuned based on increasing outcome data. Models differed according to insemination method and day of transfer. IVF models were developed using an adjustment of 1.95 h for the time taken for the sperm to fertilize the oocytes. Early cleavage stage models used morphokinetic variables t2, duration of the second cell cycle (t3–t2) and a calculated variable (t2/t5–t2). Blastocyst models were similar to the published aneuploidy risk classification model using tSB and duration of blastulation to rank embryos using the calculated variables cc2 (t3-t2), cc3 (t5-t3), dB (tB [time to blastulation from insemination]-tSB). A Wallace (UK) embryo transfer catheter under ultrasound guidance was used for embryo transfer.

Statistics

Descriptive statistics were calculated to summarize the clinical data available for the cycles in each treatment arm (EmbryoScope/standard). For the binary and categorical variables, cross-tabulations of the frequencies compared with treatment were produced. For the count variables, summary statistics (including measures of central tendency and dispersion) split by treatment were calculated. Continuous summaries of patient age, donor age (where applicable) and patient age by donor status were also calculated for completeness. A mixed-effects logistic regression model (Agresti, 2013; Jiang, 2007) was fitted to the live birth event outcome, modelling the probability of the binary outcome as a function of the explanatory variables. To account for the fact that multiple treatment cycles were observed for individual patients included in the study, a patient random effect was included in the model. Generalized linear models assume that observations are independent, whereas results from the same patient will be related. To account for the fact that multiple treatment cycles were observed for individual patients included in the study, a patient random effect was included in the model. This appropriately accounts for non-independence by modelling the correlation among patients' multiple outcomes, preventing an artificial increase in the sample size. To explicitly control for differences in the patient populations between the treatment arms, potential confounding variables from the available clinical data were also considered as other explanatory variables in the model (as fixed effects). To choose which of the available variables to include as fixed effects in the model, a stepwise variable selection procedure was undertaken. Starting with an initial model that included just the patient effect, new explanatory variables were added one at a time to look for the model that has the best value of the Akaike information criterion. This stepwise selection procedure carries on adding variables until no further improvement in the Akaike information criterion (a measure of model fit that has a penalty term for the number of parameters in the model) can be achieved (**Table 2**).

A number of interactions that allow the level of one or more variables to change the effect of another variable were made available to the variable selection procedure. Any combination of variables may

Table 2 – Variables offered to the model during the stepwise selection procedure. Those marked Y were included in the final model.

Variable	Included?
Embryoscope (y/n)	Y
Patient age (<38/38+), years	Y
Day of embryo transfer	Y
Numbr of embryos transferred	Y
Patient type (standard/oocyte share donor/ donor recipient)	Y
Donor age (where applicable)	Y
Total previous cycles	Y
Total number of previous live births	Y
ICSI (y/n)	N
Total number of miscarriages	Y
Intralipid (y/n)	Y
Duration of infertility	Y
Total number of ectopics	N
BMI (underweight/healthy/overweight/obese/ extremely obese/unavailable)	N
AMH (low/normal/high/very high/unavailable)	N
AFC (extremely low/low/somewhatlow/normal/high/ very high/unavailable)	Y
Gonadotrophin type (other/Gonal-F/Menopur/unavailable)	Y
Gonadotrophin dosing days (<10/10 to 11/12 + /unavailable)	Y
Gonadotrophin total dose (<1800 iu/1800 to 2699 iu/ 2700 to 3599 iu/3600 iu +/ unavailable)	Y
Number of eggs collected	N
Numbr of eggs inseminated	N
Ratio of metaphase 2 (mature) to total eggs	N
Number of eggs fertilized	Y
Patient age/donor recipient interaction	Y
Day of embryo transfer/number of embryos transferred interaction	Y
Embryoscope/patient age interaction	Y
Embryoscope/donor recipient interaction	Y
Embryoscope/patient age/donor recipient interaction	Y
Embryoscope/donor age interaction	N
Embryoscope/day of embryo transfer interaction	N
Embryoscope/number of embryos transferred interaction	N
Embryoscope/day of embryo transfer/number of embryos transferred interaction	N

AFC, antral follicle count; AMH, anti-Müllerian hormone; BMI, body mass index; ICSI, intracytoplasmic sperm injection.

interact with each other; therefore, as the number of potential interactions is enormous, we limited the available interactions to the following: patient age with donor recipient status, to allow for the effect of the patient's age to vary with whether they are an oocyte recipient; the day of embryo transfer with the number of embryos transferred, allowing the effect of the number of embryos to vary by transfer stage; the EmbryoScope variable, i.e. treatment effect, with each patient's age, oocyte recipient indicator, donor age (where applicable), day of embryo transfer and the number of embryos transferred; three-way interactions between EmbryoScope, recipient and patient age, and between EmbryoScope, day of embryo transfer and the number of embryos transferred. These were included as the corresponding two-way interactions without EmbryoScope were included. Importantly, these interactions were included to allow for the possibility that the size and direction of the EmbryoScope effect varied for different groups of patients.

The model-estimated effect sizes (presented as odds ratios that describe the relative difference in the odds of a live birth between different treatment cycles) are accompanied by confidence intervals that quantify the uncertainty in the odds ratio estimates arising from the sample data. The confidence intervals are constructed using case bootstrapping to achieve appropriately conservative estimates for the statistical significance of the effects of interest and sensible estimates for their uncertainty, where no analytically tractable formulas are available for the standard errors (Booth, 1995; Carpenter and Bithell, 2000; Thai et al., 2013). The observed data are resampled (with replacement) to obtain a large number of pseudo datasets (n = 1000). For each pseudo-dataset the final model is refitted and the effect size of interest calculated. The result is a large sample of estimates of the effect size that mimics the variability that we would expect to observe if we were to repeat the study many times on new sets of similar patients. The confidence interval is derived from the percentiles of the sample of estimates.

Finally, likelihood ratio tests were carried out for each of the explanatory variables that were considered but not retained in the model, after the stepwise variable selection procedure. These tests evaluate the null hypothesis that including the variable in question does not improve the model fit as measured by the likelihood.

The statistical software package R version 3.3.1 (R Core Team, 2016) was used for analyses. The *lme4* package was used to implement the generalized linear mixed effects modelling (Bates et al., 2015).

Results

The summary statistics for each clinical variable considered in the analysis versus treatment (standard/embryoscope) are shown in **Table 1**; the variables selected for inclusion in the final model are presented in **Table 2**. The interactions between the EmbryoScope and each of donor age, day of embryo transfer, number of embryos transferred, and the three-way interaction between EmbryoScope, day of embryo transfer and number of embryos transferred were omitted after carrying out the likelihood ratio tests for each, which returned a *P*-value larger than 0.1 for each of these cases, indicating no evidence to reject the null hypothesis (**Table 3**); hence, no evidence was found that the EmbryoScope morphokinetic algorithm effect varies with donor age, day of embryo transfer, the number of oocytes recovered in total, as a ratio of mature (metaphase 2) to total or the number of embryos transferred. In each case, however, the corresponding main effects were included in the model, as these variables did affect the incidence of live birth events.

Table 3 – *P*-values returned by likelihood ratio tests for including the listed variables in the model.

Variable	LRT *P*-value[a]
ICSI (y/n)	0.616
Total number of ectopics	0.595
BMI (underweight/healthy/overweight/obese/extremely obese/unavailable)	0.465
AMH (low/normal/high/very high/unavailable)	0.153
Embryoscope/donor age interaction	0.476
Embryoscope/day of embryo transfer interaction	0.553
Embryoscope/number of embryos transferred interaction	0.179
Embryoscope/day of embryo transfer/number of embryos transferred interaction	0.125
Number of eggs collected	0.245
Number of eggs inseminated	0.458
Ratio of metphase II (mature) to total number of eggs	0.257

[a] $P > 0.1$ indicate that there is no evidence to reject the null hypothesis.
AMH, anti-Müllerian hormone; BMI, body mass index; ICSI, intracytoplasmic sperm injection; LRT, likelihood ratio test.

The estimated odds ratio for patients younger than 38 years using their own eggs is 1.19, with 95% CI of (1.06 to 1.34), indicating strong evidence that the use of the EmbryoScope with the morphokinetic algorithm in this group is more likely to result in a live birth than standard treatments. The mean (SD) and median (range) for patient age for standard incubation were 35.5 (5.17) and 36.0 (19–54); and for Embryoscope 36.7 (4.80) and 37.0 (22–50), respectively. The total data were 35.6 (5.15) and 36.0 (19–54), respectively.

EmbryoScope treatment in which a single blastocyst is transferred was compared with standard treatment in which two blastocysts were transferred, which needed to take account of the effect of the number of embryos transferred. For the age group younger than 38 years using their own eggs, there is some evidence that transfer of a single blastocyst after EmbryoScope is associated with fewer live births than transfer of two blastocysts after standard treatment (OR 0.854; 95% CI 0.735 to 1.000).

For non-recipients in the group aged 38 years and over, there is strong evidence that transfer of a single blastocyst after EmbryoScope is associated with fewer live births than transfer of two blastocysts after standard treatment (OR 0.603; 95% CI 0.478 to 0.748). For recipients in the group aged 38 years and over, no evidence was found of a difference between transfer of a single blastocyst after EmbryoScope and transfer of two blastocysts after standard treatment (OR 0.981, 95% CI 0.557 to 1.750). These data are important, especially in the context of multiple pregnancies in which the data demonstrated a highly elevated risk of multiple pregnancy in all groups when two embryos where transferred (**Table 4**).

Discussion

In summary, the results demonstrate an 19% increase in live birth rate with EmbryoScope and morphokinetic algorithm embryo selection for patients younger than 38 years, and a 37% increase in live birth rate for recipients aged over 37 years. Recipients aged over 37 years did equally well in live birth outcome, with only a single blastocyst

Table 4 – Multiple birth in relation to age, stage of transfer and age of female.

	Standard (♀ <38)	Standard (♀ >37)	Time-lapse (♀ <38)	Time-lapse (♀ >37)
Single embryo transfer (%)	0.8	1.5	0.7	0.0
Double embryo transfer (%)	31.1	24.2	30.7	26.7
Single blastocyst (%)	0.8	1.5	0.9	0.0
Double blastocyst (%)	38.3	34.7	40.9	35.0

from the EmbryoScope compared with two after standard treatment. The gain of increased incidence of live birth by transferring two embryos needs to be balanced against the increased risk of a multiple pregnancy by about 30– 40%. It is recognised, however, that one of the limitations of such comparative studies is the distinction of the use of algorithms generated by TLI compared with any benefits accruing from the sole use of closed incubation systems; and conversely the use of algorithms of morphokinetic development derived in open culture systems. No adequate study has yet been conducted to evaluate and distinguish these possibilities fully.

The introduction of time-lapse imaging is a recent innovation in IVF (Hlinka et al., 2012a, 2012b; Lemmen et al., 2008; Meseguer et al., 2011), especially since the development of wholly enclosed systems. The key theoretical benefits of using time lapse in human IVF treatment are twofold: providing images, not specifically related to kinetics, that permit assessment of human embryos in unparalleled detail within small (10–20 min) time frames, without disturbing the controlled culture environment (closed system) (Campbell and Fishel, 2015); and to provide detailed morphological and its related kinetic information. The value of both elements of time-lapse in clinical outcome are currently being assessed by many groups. Cruz et al. (2012), working with donor oocytes, used the EmbryoScope time lapse incubator (Vitrolife, Sweden) to compare clinical outcome between standard incubators and the closed incubation provided by the EmbryoScope, without consideration of the additional 'morphokinetic' information acquired and selection of embryos based solely on conventional morphological criteria. Embryos were randomly distributed between the incubators, and the authors reported no differences in their development, blastocyst viability or ongoing pregnancy rate. More recently, a randomized controlled trial (Park et al., 2015) confirmed no significant difference in the mean number of good-quality embryos or ongoing pregnancy rates when comparing closed and conventional incubation, although this study was limited by the short incubation time and day 2 transfer.

Such technology has captured the imagination of practitioners as both a research tool (Costa-Borges et al., 2016; Hardarson et al., 2015; Mölder et al., 2015), comparing embryos resulting from various clinical conditions (Balakier et al., 2016; Gurbuz et al., 2016; Hashimoto et al., 2016; Kaihola et al., 2016; Lindgren et al., 2016), and as a potential prognosticator in clinical practice (Adamson et al., 2016; Chen et al., 2016; Kong et al., 2016; Liu et al., 2016; Milewski et al., 2015; Mizobe et al., 2016; Rubio et al., 2014; VerMilyea et al., 2014; Wu et al., 2016a, 2016b; Yang et al., 2014), although some studies negate this (Freour et al., 2015; Wu et al., 2016a, 2016b). Bronet et al. (2015) even found distinctive morphokinetic differences between male and female embryos. A few studies have purported to relate discriminating morphokinetics of euploid and aneuploid embryos (Campbell et al., 2013a, 2013b; Minasi et al., 2016; Vera-Rodriguez et al., 2015), although this has been disputed (Rienzi et al., 2015). Much complexity arises in the analysis of embryo morphokinetics and attempting to compare such with clinical outcome. It is important to have consistent ambient conditions for embryo cleavage, but most studies ignore patient and treatment variables that have an effect and are so vital to meaningful analyses (Kirkegaard et al., 2016); some studies also only consider morphokinetics as a prognosticator for blastulation (Conaghan et al., 2013). Kirkegaard et al. (2014) showed that about 50% of embryos classified as non-usable were in fact viable; and further studies have shown such an approach to be no better than morphology assessment (Kieslinger et al., 2016). Other studies are inadequate in relation to number of cases, comparative controls, including the assessment of quality control for annotation, and end-points (Wu et al., 2016a, 2016b). Much of this has been discussed in an excellent review by Kaser and Racowsky (2014).

In clinical practice, it is incumbent on practitioners to continue to improve protocol according to new data. During this period of analysis, it was necessary to use an evolving series of selection models, by which the transferred population is continuously narrowed, in morphokinetic terms, such that the ratio between positive and negative known implantation data may reach a plateau, as well as the area under the receiver operator characteristic curve, commonly used to measure the prognosticative power of a model. When each model was derived, no assumptions were made about the morphokinetic variables to be used. Information was used to derive the models and 'holdback data', not used in model derivation, was used to test before introducing prospectively.

As far as is known, the data presented here are the largest to date investigating the use of TLI algorithms for embryo selection and its effect on the incidence of live birth, with comprehensive morphokinetic variables assessed during embryo cleavage and in relation to other patient and treatment-related factors. It is highly probable that the increasing incidence of aneuploid embryos with age (Lee et al., 2015) has a negating effect of discriminatory morphokinetic factors in woman aged over 37 years.

As the ploidy status of human embryos is critical to successful outcome, early clinical studies were undertaken with TLI to assess any correlation between embryo aneuploidy and morphokinetic parameters. The studies by Campbell et al., (2013a, 2013b) and Campbell (2014) indicated the possibility of ranking embryos in relation to ploidy, but such hierarchical data are only risk-based. Only by evaluating chromosome copy number directly using invasive biopsy procedures would the most accurate knowledge of embryo ploidy be provided. Such studies comparing embryo ploidy and morphokinetics, however, are still fraught with complexity, not least the type of aneuploidy be it one compatible with full-term delivery such as trisomy 21; to those not compatible with implantation, such as many monosomies; and complex versus simple aneuploidy, mosaicism and segmental aneuploidies may well have additional effects on morphokinetics. Additionally, simply having closed systems throughout cleavage *in vitro* as distinct from any morphokinetic differential may have a beneficial effect.

Kong et al. (2016) using time-lapse imaging reported a relationship between early cell division behaviour and developmental potential with elongation or shortening of the cell cycle affecting cell number, respectively. This study concluded that, by excluding such embryos,

the incidence of implantation and live birth after day 3 transfer of embryos increased when the cell number of the embryo was maximal.

In assessing the limitations of this study, the EmbryoScope is a closed system for embryo culture and in itself may procure benefit; this was not assessed. Given that patient factors and some treatments *per se* affect embryo morphokinetics, not all possibilities have been fully explored. In the pragmatic world of patient treatment, we were unable to use morphokinetic data as a blinded randomized control study. Patients were not randomly allocated to EmbryoScope or standard incubation and selection. It is not possible to account for any differences not contained within the available data, and therefore elimination of all confounding effects in a non-randomized study can never be guaranteed. Larger studies using donor oocyte recipients are needed to further evaluate the findings here. The evaluation is based on live birth per embryo transfer to focus on the value of morphokinetic data. Embryo implantation and successful progression to live birth also requires a receptive endometrium and functional placenta.

In summary, many factors feed into embryo morphokinetics that need to be controlled for, and data need to be presented on live birth outcome with sufficiently powered studies that take into account patient and treatment factors. Ideally, prospective randomized controlled studies are needed, but pragmatic difficulties exist in undertaking such studies, not least to obtain sufficient patient recruitment, and for prospective annotation data to be ignored. The present study used morphokinetic data for selection of embryos for transfer; a 19% increase in live birth was observed for women younger than 38 years using their own oocytes. The advantage of the EmbryoScope morphokinetic data was evident too for recipients aged over 37 years, demonstrating that the transfer of a single blastocyst had the same chance of a live birth as two transferred after standard treatment without the risk of multiple pregnancy. Finally, it must always be recognized that such analyses cannot account for all factors affecting successful pregnancy outcome, particularly in the IVF population cohort; normal placental function (Fishel et al., 2016) and endometrial receptivity (Miravet-Valenciano et al., 2015; Nejat et al., 2014) are also essential.

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Perivitelline threads in cleavage-stage human embryos: observations using time-lapse imaging

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KEY MESSAGE
Perivitelline threads are observed during human zygote cleavage. Spanning the perivitelline space, they appear as the cytoplasmic membrane withdraws from the zona pellucida. They are observed in 56% of embryos, and are associated with fragmentation. We postulate that they have a role in the mechanics of cytokinesis and fragmentation.

A B S T R A C T

Time-lapse imaging of the human preimplantation embryo *in vitro* has revealed a transient phenomenon involving the appearance of perivitelline threads, commonly observed at the two-cell stage. These threads span the perivitelline space, arising at the specific area where the cytoplasmic membrane contacts the zona pellucida, before any perivitelline space is formed. The threads persist as the cytoplasmic membrane retracts from the zona pellucida to form the first cleavage furrow. In this observational report, these structures and their incidence are described. A total of 834 time-lapse videos from IVF treatment cycles, one per patient, were retrospectively analysed for perivitelline threads, from pronuclear formation until completion of the first cell cycle. Threads were observed in 56.4% (470/834) of embryos and varied from a single to an array spanning an area of the zona pellcida. A total of 91.9% (432/470) were seen to form after cytoplasmic membrane–zona-pellucida contact. A total of 76.4% (359/470) were visible at the first cleavage furrow; 77% (362/470) were associated with cytoplasmic fragments at the two-cell-stage. Presence or absence of threads did not affect embryo development. This descriptive study is limited; further characterization of these structures is needed to elucidate their potential role in early human embryo development.

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Introduction

Communication between the oocyte and its surrounding granulosa cells is vital during maturation. To allow gap junction communication to occur, the zona pellucida is pierced by specialized granulosa trans-zonal projections (TZP) that reach the oolemma (Gilchrist et al., 2008). Like filopodia, they can be rich in F-actin, or composed primarily of microtubules (Li and Albertini, 2013). The reduction of these processes as the oocyte matures may contribute to the formation of the perivitelline space (PVS) (Inoue et al., 2007). The PVS of the oocyte contains a hyaluronan-rich extracellular matrix, not visible with light microscopy. The size of the PVS is significant, affecting the incidence of polyspermy, fertilization rate and pronuclear morphology (Rienzi et al., 2005; Yoshida and Niimura, 2011).

In many species, the orientation of the first cell division depends on the positioning of the mitotic spindle relative to an axis of polarity (Siller and Doe, 2009) (Lu and Johnston, 2013). In the human, no direct data are available on the mechanisms involved in polarity establishment or division plane orientation during cleavage; however, it is likely that this is governed by interactions between the cytoskeleton, proteinase-activated receptor signalling and cell-to-cell communication analogous to those reported in other species (Ajduk and Zernicka-Goetz, 2016).

In early embryonic development, the appearance of fragmentation is well known to affect embryo quality, but the mechanism of fragmentation is not fully understood. Hypotheses concerning the factors controlling fragmentation have included telomere length, maternal age, association with lipoproteins and reactive oxygen species (Fujimoto et al., 2011). Previous time-lapse imaging studies have shown that most fragments appear at the two-cell-stage, and some can be resorbed and disappear in the next cleavages (Mio and Maeda, 2008).

In this time-lapse imaging study, the incidence of perivitelline threads in human embryos and their association with the first cleavage furrow, fragmentation, embryo ploidy and implantation potential is investigated.

Materials and methods

Data for this research were obtained from 834 treatment cycles across two CARE Fertility IVF clinics (CARE Fertility Nottingham and CARE Fertility Tunbridge Wells), using the same laboratory operating procedures between 11 November 2014 and 1 December 2016. All operating procedures and protocols complied with UK regulations (The Human Fertilisation and Embryology Act, 1990, 2008). The study did not require ethical or institutional review board approval, as it was conducted according to previously validated procedures. This was a retrospective observational study, blinded to embryo developmental or clinical outcome, using time-lapse imaging (EmbryoScope™, Vitrolife, Sweden) for the recording of fertilization, presence, appearance and dynamics of threads, and the morphokinetics of early cleavage events.

Patient criteria

For each of the 834 fresh clinical IVF or intracytoplasmic sperm injection (ICSI) cycles, a time-lapse video, representing a single embryo per patient, was randomly selected by the roll of a die, which then dictated the number of culture well to assess, regardless of fertilization status or embryo fate. Each embryo was assessed from pronuclear formation until completion of the first cell cycle. Female patient age ranged from 24–47 years, and mean female age was 35.98 (±4.36 SD).

Ovarian stimulation

Pituitary suppression was achieved with a gonadotrophin-releasing hormone agonist (Suprecur® 0.5 ml subcutaneously daily; Sanofi Aventis, UK) or antagonist (Cetrotide 0.25 mg daily; Merck Serono, UK). For ovarian stimulation, recombinant FSH (Gonal-F, Merck Serono, UK), human menopausal gonadotophin (Menopur, Ferring, UK), or both, were used. Dosage of FSH ranged from 150 IU to 600 IU per day according to individual patient response.

Oocyte retrieval and embryology

The methodology for oocyte retrieval, insemination and embryo culture using the EmbryoScope™ (Vitrolife, Sweden) has been described previously (Campbell et al., 2013). In brief, after retrieval and insemination, oocytes were placed individually in microwells of the EmbryoSlide, loaded into the EmbryoScope™ on day zero for ICSI (after injection) and on day 1 for IVF embryos and cultured until day 5 or 6 of development. Time-lapse images of each oocyte, with at least a single pronucleus, were acquired every 10 min through seven focal planes on the Z axis, 15 µm apart. EmbryoScope™ images were acquired by a 12-bit monochrome complementary metal-oxide semiconductor camera and a custom-made Hoffman modulation contrast with a X20 objective, giving a resolution of 3 pixels per µm and images between 800 × 800 to 1000 × 1000 pixels (scaled to 800 × 800 pixels for export).

The time-lapse images were used for assessing pronuclei, threads and embryo development. Selected embryos were transferred into the uterus, cryopreserved or discarded, as per standard clinical protocol. Images were annotated (described and recorded by time and embryo stage at observation) using the EmbryoViewer® image analysis tools and software by embryologists with demonstrated competency.

Preimplantation genetic screening

Preimplantation genetic screening was carried out using trophectoderm biopsy on day 5 or day 6 and next-generation sequencing. Amplified samples for next-generation sequencing were processed with VeriSeq PGS kit (Illumina). Blastocysts were vitrified after biopsy and warmed on the same day as embryo transfer.

Assessment and description of perivitelline threads

The presence or absence of threads was determined by carefully focusing through all Z-plane images from the time of pronuclear formation until completion of the first cytokinesis. The time-lapse images of the randomly selected embryos were retrospectively studied, and the following details of the threads were annotated: time of appearance, abundance, relationship to the zona pellucida, association with fragments and location. Embryologists conducting these annotations were blinded to downstream developmental fate or clinical outcome. Annotations and definitions associated with threads are presented in **Table 1**.

Table 1 – Annotation of perivitelline threads.

Category for annotation	Annotations and definitions		
	Yes	No	Inconclusive
Presence of perivitelline threads (presence was further classified by number. (**Figure 1**)	If at least one perivitelline thread observed. If present, threads classified by quantity	When perivitelline space visualized with no threads observed.	Perivitelline space obstructed by fragmentation, visibility obscured by embryo orientation or position in well/ well shadowing.
Originate from contact area between cytoplasmic membrane and zona pellucida.	Perivitelline threads arise from an observed area of contact between cytoplasmic membrane and zona pellucida.	Perivitelline threads observed following no prior contact between cytoplasmic membrane and zona pellucida.	Images obscured as described above. Contact area may be out of focal plane.
Association with fragments	Perivitelline threads connected to fragments, which localize to region of the perivitelline space where the threads are first observed.	Perivitelline threads observed with no association to fragments. Either fragments not present or observed without threads attached.	Images obscured as above, or high degree of fragmentation precludes visibility .
Association with cleavage furrow	Perivitelline threads clearly observed with first cleavage furrow connecting the invagination of the cleavage furrow to zona pellucida.	Perivitelline threads distinct from point of cleavage furrow. Appear to originate elsewhere along the cytoplasmic membrane.	As previously described above or orientation of cleavage in different focal plane. i.e. Perpendicular to camera position.

Annotation criteria and classification of perivitelline thread appearance

Threads were identified as present or absent and, if observed, they were categorized according to number as follows: single or double threads (class I); a group of three to five threads (class II); or an array of many threads spanning a large surface area of the zona pellucida (class III) (**Figure 1**).

In cases in which threads were positively identified, it was ascertained whether they appeared after cytoplasmic membrane–zona pelluicida contact, whether they were located at the cleavage furrow, and whether they were associated with fragmentation.

Assessment of thread–cleavage furrow association was considered positive if threads appeared to connect the invagination of the cytoplasmic membrane with the zona pellucida, becoming more prominent as the cleavage furrow deepened.

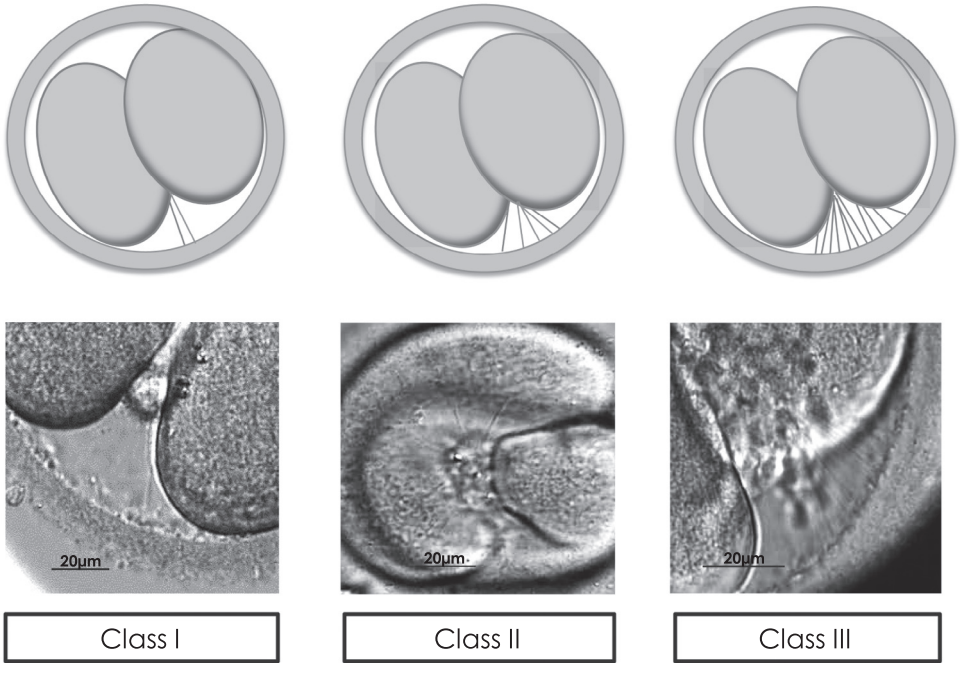

Figure 1 – Classification of perivitelline threads, with timelapse image examples. Threads were classified into three groups, based on their quantity: one or two individual threads (I), three to five threads (II) and more than five threads (III). Class III included observations of large arrays spanning a high proportion of the internal zona.

Association with fragmentation was indicated when threads appeared to be physically connected to cytoplasmic fragments at any time during their appearance.

The degree of fragmentation was routinely assessed and recorded by an embryologist; competency was assessed for this task before the retrospective analysis of the time-lapse videos, using the software of the time-lapse incubator. According to standard operating procedure, fragmentation was annotated only when cytokinesis was complete, and the two blastomeres were rounded and fully formed. The degree of fragmentation was expressed as 0–10%, 10–20%, 20–50%, and over 50%.

In all cases, where the time-lapse images did not result in a clear view of the cytoplasmic membrane–zona pellucida contact point and first cleavage furrow sufficient to identify the presence or absence of threads, the embryo was scored as 'inconclusive'. Inconclusive observations were possibly due to the transient nature of the threads, and limitations of the 10-min image frequency. Therefore, definitive absence of threads could not be determined. For a similar reason, in some cases, formation of the cleavage furrow was not captured. Observations were also considered inconclusive if the first cleavage plane orientation or shadowing caused by an off-centre position in the culture well, hindered visibility of the perivitelline space.

Measurement of perivitelline threads

Measurements were taken of the longest thread in each of 50 embryos, randomly selected from the original data set, using time-lapse imaging software. The measurements represent 'snap-shot' values, as the threads appeared to be dynamic structures showing extension and retraction.

Outcome measures

Insemination type, ploidy status, early morphokinetics, final disposition and implantation data of embryos were analysed. Implantation rate is reported for embryos with known implantation data (KID). That is, only transferred embryos where the fate was traceable to an individual embryo were included. Implantation was confirmed by the presence of a fetal heart beat at ultrasound scan at 7 ± 1 week gestation. Implantation rates for KID embryos were calculated as follows: (number of implanted KID embryos /total number of KID embryos) × 100%.

Statistical analysis

Statistical significance was determined using chi-square 2 × 2 analyses. $P < 0.05$ was considered significant.

Results

Classification of perivitelline threads

Using a classification system to describe the number of threads observed (**Figure 1**), the three classes were detected in similar frequencies; class I threads were observed in 35.2% (165/469) of embryos analysed, compared with 38.6% (181/469) of class II and 26.2% (123/469) displaying class III threads. One embryo with threads could not be classified accurately owing to the orientation of the cleavage furrow and was excluded from this data set.

Table 2 – Incidence of perivitelline threads: insemination type and pronuclear status.

	Perivitelline threads observed (% of total)	No Perivitelline threads observed (% of total)	Inconclusive (% of total)
Insemination type			
IVF (*n* = 134)	71 (53.0[a])	30 (22.4)	33 (24.6)
ICSI (*n* = 700)	399 (57.0[a])	153 (21.9)	148 (21.1)
Pronulclear status			
1PN (*n* = 47)	27 (57.4[a])	9 (19.1)	11 (23.4)
2PN (*n* = 762)	428 (56.2[a])	168 (22.0)	166 (21.8)
3PN (*n* = 21)	11 (52.4[a])	6 (28.6)	4 (19.0)
4PN (*n* = 4)	4 (100.0)	0	0

ICSI, intracytoplasmic sperm injection.
[a] No significant difference.

Incidence and association of perivitelline threads

Of the 834 randomly selected embryos annotated, threads were visible in 56.4% (470/834) of embryos, not visible in 21.9% (183/834), and inconclusive in 21.7% (181/834).

A total of 134 embryos observed in this study were derived after IVF insemination, and 700 from ICSI. No significant difference was found in the incidence of threads between insemination methods or pronuclear status of the embryos included in this study (**Table 2**).

The fate of the 762 normally fertilized embryos available for clinical treatment was investigated for the apperance of threads. The proportions of embryos in which threads were seen and transferred, vitrified or discarded were 56.9%, 61.8% and 53.1%, respectively. Transferred embryos were of varying quality; however, only top-quality blastocysts were vitrified. No significant difference was observed in the occurrence of threads between vitrified embryos (top quality) and discarded embryos (not suitable for vitrification) (**Table 3**).

Table 3 – Fate of 762 two pronuclei embryos.

	Perivitelline threads observed (% of total)	No Perivitelline threads observed (% of total)	Inconclusive (% of total)
Transferred total (*n* = 283)	161 (56.9)	72 (25.4)	50 (17.7)
Vitrified (top-quality blastocysts) total (*n* = 157	97 (61.8[a])	29 (18.5)	31 (19.7)
Discarded (poorer quality blastocysts) total (*n* = 322)	171 (53.1[a])	67 (20.8)	84 (26.1)

[a] No significant difference.

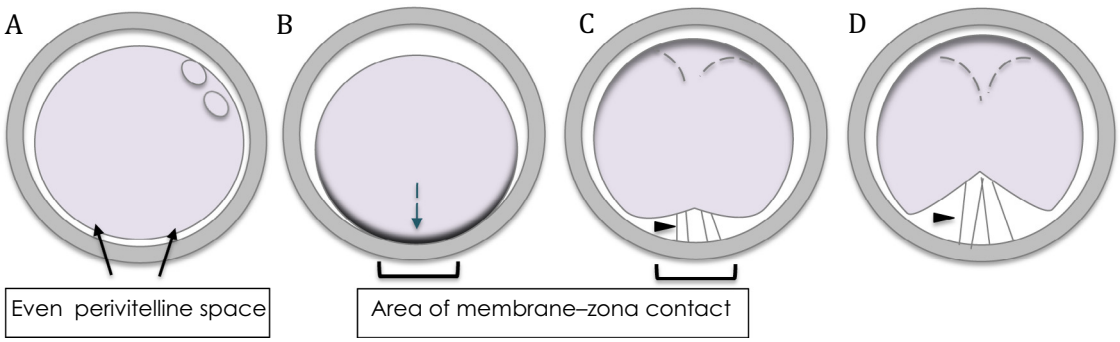

Figure 2 – The sequence of events leading to the appearance of perivitelline threads. When the zygote is positioned centrally within the perivitelline space (A) no perivitelline threads are visible; (B) the zygote moves towards the inner edge of the zona pellucida (dashed arrow) making proximal contact; (C) as cytokinesis occurs the threads appear in the newly formed perivitelline space as the embryonic membrane withdraws from the contact area (arrow head); (D) perivitelline threads are seen to extend as cytokinesis progresses.

Zona pellucida–cytoplasmic membrane contact and perivitelline thread appearance

Perivitelline threads appeared from the region of the cytoplasmic membrane–zona pellucida contact area. This is the region of the zygote, observed before first cleavage, where no PVS exists and the cytoplasmic membrane appears to touch the inner zona pelluicida. As the zygote retracted from the zona pellucida, at the start of cleavage, threads were observed extending between the cytoplasmic membrane and zona pellucida (**Figure 2**, **Figure 3** and **Supplementary Video 1**). This association was seen in 91.9% (432/470) of embryos, whereas 4.3% (20/470) did not show such association and 3.8% were inconclusive (18/470).

Threads were observed in 76.4% (359/470) of embryos specifically at the invagination of the first cleavage furrow. In 10% (47/470) of embryos, threads were observed in other areas of the developing perivitelline space, not in association with the furrow. A total of 13.6% (64/470) were considered inconclusive. Threads were also observed

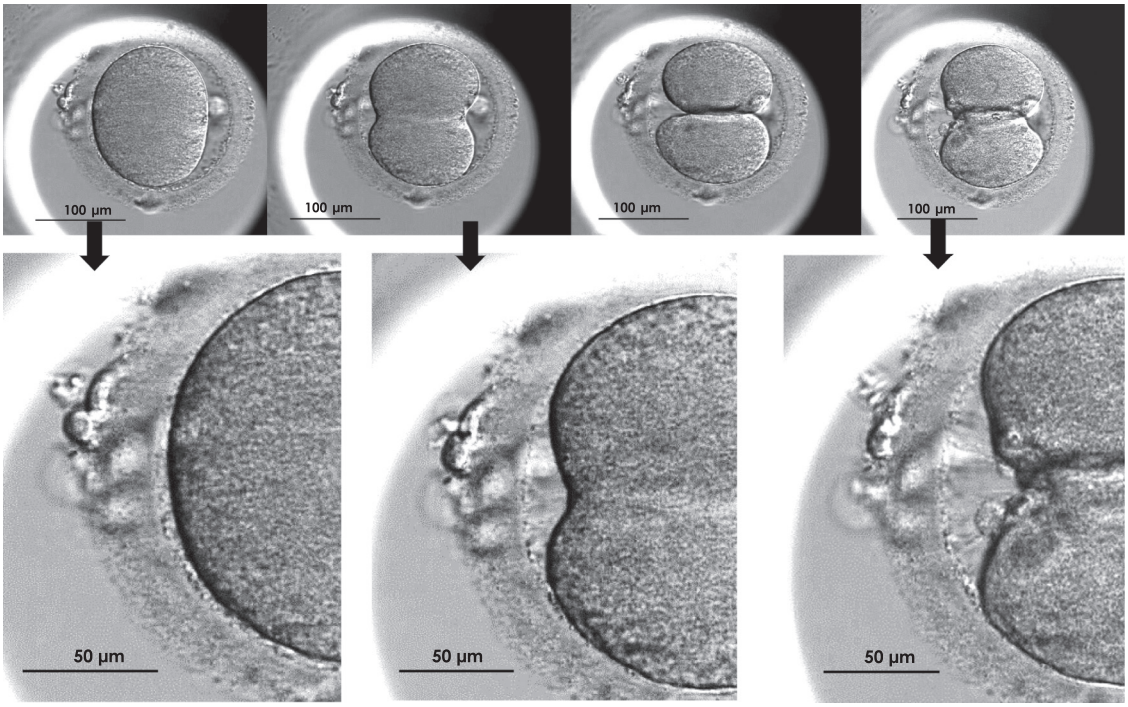

Figure 3 – Perivitelline threads observed in the perivitelline space as the cytoplasmic membrane withdraws from the zona pellucida. Perivitelline threads appear to follow the line of the cleavage furrow and extend as the membrane moves into the furrow (Supplementary Video 1).

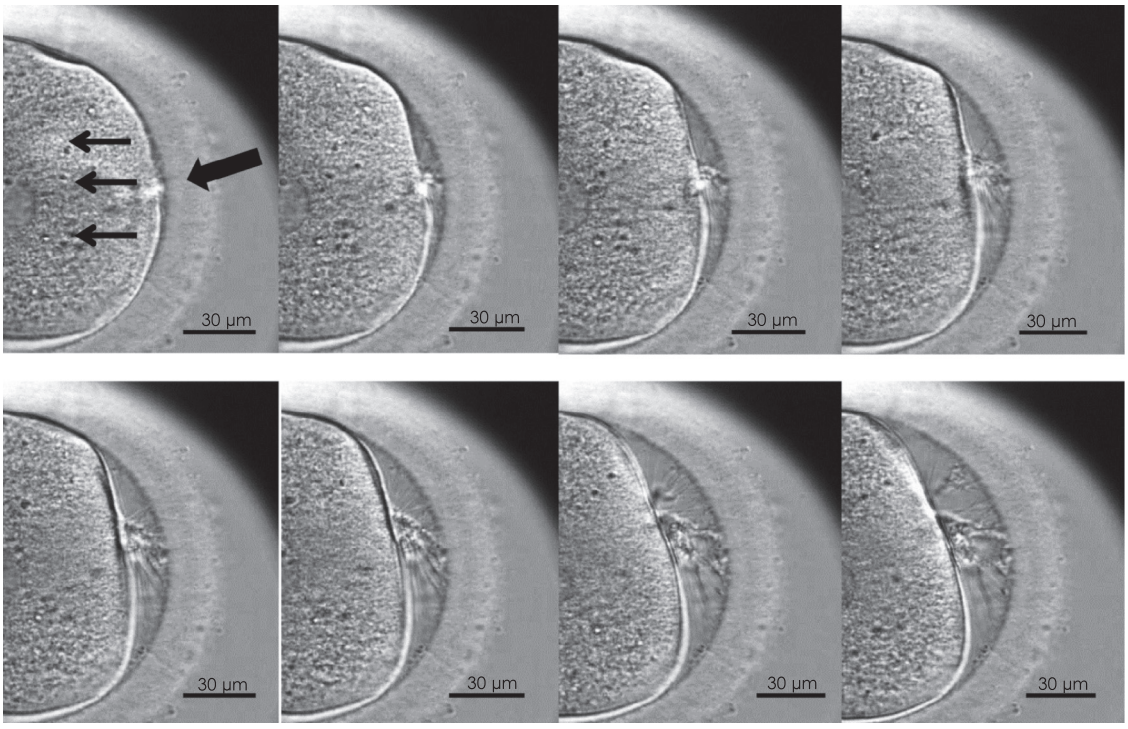

Figure 4 – Before pronuclear fading, and the first cleavage, the membrane withdraws from close proximity to the zona pellucida (black arrows), and an array of perivitelline threads appears, connecting the membrane to the zona pellucida, elongating with time. (Supplementary Video 2b).

before the first cleavage event (**Figure 4**): a small number of zygotes were seen to have a 'ripple' or 'wave' of threads along the inner zona pellucida creating transient regions of contact between the membrane and zona pellucida (**Figure 5**) (**Supplementary Videos 2a and 2b**).

Perivitelline threads and fragmentation

Fragmentaion was observed in 470 embryos. Perivitelline threads were positively associated with the appearance of fragments in 77% (362/470) of these embryos, regardless of pronuclear status. In 15.5%

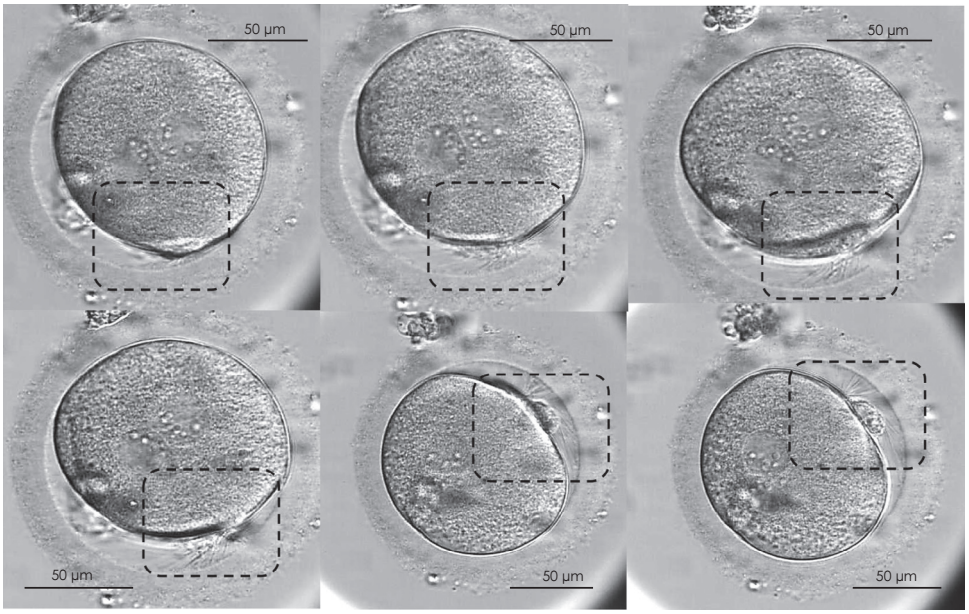

Figure 5 – An array of perivitelline threads appear as a 'wave' in the perivitelline space, formed in association with the zygote cytoplasmic membrane (Supplementary Video 2a).

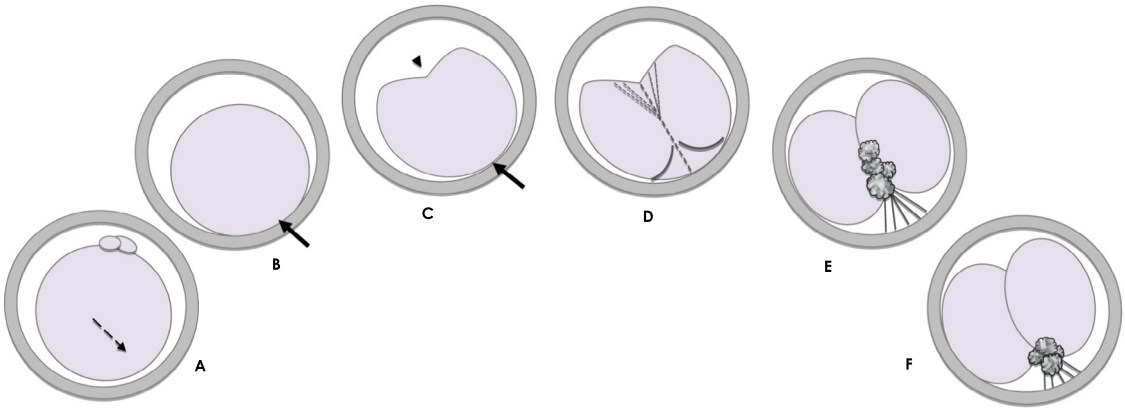

Figure 6 – (A) Observation of perivitelline threads and their association with fragmentation and cleavage orientation. After pronuclear fading, the zygote moves towards the inner surface of the zona pellucida (dashed arrow) to form a direct contact area (B, arrow). The cleavage furrow is initiated from the most distal region of the zygote (C, arrow head), directly opposite this area of contact (arrow). Perivitelline threads are thought to extend along this axis, directing cleavage in a 'purse string' effect drawing the distal cytoplasmic membrane inwards towards this area of membrane – zona pellucida contact (D). As the cleavage furrow deepens the perivitelline threads are observed in the perivitelline space (E). As cytokinesis progresses, the threads are observed attached to extra-embryonic cytoplasm, which is drawn out as fragments (E and F).

Figure 7 – Magnified images of the cleavage furrow in association with fragments and perivitelline threads. Fragments appear in a purse-string like manner (Supplementary Video 3a).

(73/470) of the fragmented embryos, the cleavage furrow orientation prevented clear assessment of threads, so these were scored 'inconclusive'.

In 7.4% (35/470) of embryos with fragmentation, no association was found between thread and fragment appearance. Of the 470 embryos in which threads were observed in the perivtelline space, 3.0% (14) showed no fragments.

After pronuclei fading, the zygote typically shifts within the perivtelline space, coming into contact with the zona pellucida (**Figure 6B**, black arrow). The cleavage furrow was observed to initiate in the region directly opposite this area of contact (**Figure 6C,** small arrow head). Threads appeared to extend along this axis, seemingly drawing the cytoplasmic membrane inwards towards this area of contact (**Figure 6D**). As cytokinesis progressed, the threads appeared to pull out fragments from the cytoplasmic membrane at the cleavage furrow (**Figure 6E**). The fragments did not appear to detach fully from the membranes, remaining amassed in the furrow (**Figure 7** and **Supplementary Videos 3a–3d)**.

The relationship between the number of threads (classes I–III) and the percentage of fragmentation is shown in **Table 4**. Five out of the six embryos with the highest degree of fragmentation displayed class III threads.

Threads were not observed, despite good visualization of the cleavage furrow, in 183 (21.9%) of embryos. A total of 91.8% (168/183) were normally fertilized zygotes (two pronuclei); these were assessed for degree of fragmentation and compared with those with threads (**Table 5**). No differences were observed in the degree of fragmentation among the embryos with and without threads.

Perivitelline threads and embryo ploidy

Of the randomly selected embryos in this study, 30 normally fertilized embryos had undergone preimplantation genetic screening (PGS).

Table 4 – Degree of fragmentation in each thread classification (I-III).

Degree of fragmentation (%)	Class I, *n* (%)	Class II, *n* (%)	Class III, *n* (%)
0–10	87 (33)	108 (41)	69 (26)
10–20	19 (33)	23 (40)	15 (26)
20–50	0	1	5

Table 5 – Degree of fragmentation with and without threads in 2PN embryos at two-cell stage.

Degree of fragmentation (%)	Perivitelline threads observed, *n* (%)	No perivitelline threads observed, *n* (%)
0–10	337/422[a] (79.9[b])	137/168 (81.5[b])
10–20	74/422[a] (17.5[b])	27/168 (16.1[b])
20–50	11/422[a] (2.6[b])	4/168 (2.4[b])

[a] Six embryos were not annotated for fragmentation at two-cell stage and are excluded from these data.
[b] No significant difference.

Of the 28 reported as aneuploid, 19 showed threads at two-cell-stage. This is not significantly different from the incidence of threads in the unscreened two pronuclei embryo group (56.2%) (**Table 2**). Only two embryos were euploid, both of which had threads. Five of 28 PGS embryos did not have threads and four were considered inconclusive for thread assessment.

Table 6 – Embryo morphokinetics.

	Perivitelline threads observed, *n* (% of total)	No Perivitelline threads observed, *n* (% of total)	Inconclusive , *n* (% of total)
Morphokinetic timings (h) pronuclear (PN) fade to wo-cell [t2-tPNf] 741			
1.8–2.4	159	63	48
270	(58.9[a])	(23.3)	(17.8)
2.4–3.0	166	60	62
288	(57.6[a])	(20.8)	(21.5)
>3.0	88	45	50
183	(48.1[a])	(24.6)	(27.3)
Direct cleavage to three-cell			
t3–t2 = 0	43	17	19
79	(54.4[b])	(21.5)	(24.1)

[a] No significant difference.
[b] No significant difference compared withs 0- to two-cell cleavage.

Perivitelline threads and blastocyst development

For a subset of 125 randomly selected embryos, the presence of perivitelline threads was assessed up to the blastocyst stage. A total of 58.4% of embryos (73/125) displayed threads, 90.4% were observed at first cleavage, the latest during blastocyst collapse and re-expansion. In 16.4% of embryos, threads were seen more than once during development. A total of 82.2% of threads (60/73) were associated with the appearance of fragments. Increasing cell number and fragmentation, however, made annotating beyond the two-cell-stage more challenging.

Early morphokinetics and perivitelline threads

As most threads were observed during the time of the first cleavage, the time from pronuclear fading (tPNf) to completion of the first cleavage (t2) was analysed (t2-tPNf) for the incidence of threads. No association was seen between the incidence of threads and the time from pronuclear fading to completion of the first division (**Table 6**). No significant difference was seen in the incidence of threads in 79 embryos that underwent a trichotomous first division (dividing directly from one to three cells), and those that divided into two cells (**Table 6**).

Perivitelline threads and implantation

Of all embryos transferred, embryos with known implantation status (*n* = 169) were identified from the data. For KID embryos that did not exhibit threads, the implantation rate was 40% (22/55); for KID embryos exhibiting threads, the implantation rate was 32.5% (37/114). Implantation rates for KID embryos were calculated for each of the three classes of threads (**Figure 8**).

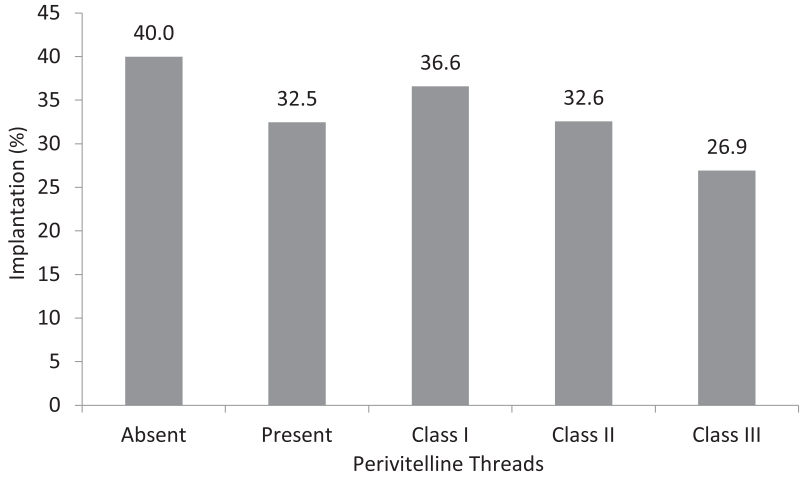

Figure 8 – Implantation rates for embryos with known implantation status stratified for embryos with (*n* = 114) and without perivitelline threads (*n* = 55) and for those with perivitelline threads for each of the classifications of threads (I–III). Class I (*n* = 41); class II (*n* = 43); class III (*n* = 46).

Length of perivitelline threads

Measurements of 50 threads gave a mean maximum length of 25.3 µm (±9.50 SD), and a range from 9–62 µm. Because of limitations of the image resolution, diameters were only estimated at less than 1 µm.

Discussion

This observational study describes structures referred to as 'perivitelline threads', that appear to span the PVS during early cleavage of many human embryos *in vitro*. They exist transiently, measuring up to 62 µm in length, seemingly connecting the cytoplasmic membrane to the zona pellucida. They present either singly or as an array, and most commonly at the site of prior cytoplasmic membrane and zona pellucida contact. Retrospective analysis of time-lapse videos in a clinical IVF setting has revealed this previously unreported phenomenon.

At present, the constitution and origin of the threads remains unknown. This study focuses on the incidence and visual description of these threads and their association with the first cleavage furrow and fragmentation.

Previously characterized structures with similar morphological characteristics as the threads reported here include filopodia and TZP. Specialized filopodia have been reported in the mouse embryo at compaction from the eight-cell stage, and spanning the blastocoel cavity connecting the trophectoderm to the inner cell mass of blastocysts (Fierro-González et al., 2013; Salas-Vidal and Lomelí, 2004). The initiation and elongation of filopodia depend on the precisely regulated polymerization, convergence and crosslinking of actin filaments (Mattila and Lappalainen, 2008). Filopodia are involved in a multitude of cellular processes including cell–cell communication, over both short and long distances (Danilchik et al., 2013; Sagar et al., 2015; Salas-Vidal and Lomelí, 2004). They have been demonstrated to facilitate the internal cellular transport of proteins, including the actin dependant migration of cortical granules to the outer plasma membrane in the oocyte at fertilization (Ajduk and Zernicka-Goetz, 2016; Antczak and Van Blerkom, 1999; Bauer et al., 2008; Cheeseman et al., 2016). It is possible that the threads reported in this study are filopodia, although this is purely speculative and based on morphology alone.

Alternatively, the threads might be remnants of the corona radiata cytoplasmic projections or TZP. The latter are known to penetrate the zona pellucida during oogenesis and provide the structural basis for more complex patterns of intercellular communication that emerge at later stages of follicle development (Carabatsos et al., 1998; Shuhaibar et al., 2015). The role of TZP is to support oocyte growth, and they are also involved in nutrition and RNA trafficking (Albertini and Barrett, 2003; Macaulay et al., 2016; Makabe et al., 2006). At least two types of TZP have been described: actin rich projections into the zona pellucida, which form gap junctions at the oocyte plasma membrane, and coiled projections, which form adherens junctions at deep invaginations at the oocyte surface (Albertini and Barrett, 2003). These structures support active organelle movement from somatic cells towards the oocyte surface. This dialogue coordinates oocyte growth and cell cycle progression.

It is possible that perivitelline threads observed in this study are TZP remnants; however, this is challenged by the fact that the threads were not observed in the PVS in oocytes after denudation, during ICSI or any other time during time-lapse incubation (data not shown). A small number of zygotes were seen to have a 'ripple' or 'wave' of threads within the PVS, creating transient regions of contact between the zygote membrane and zona pellucida (**Supplementary Videos 2a** and **2b**). This behaviour may be more akin to filopodia than TZP, and suggests that threads are not only present when a larger perivitelline space is visualized at early cleavage.

Stringent restrictions on human embryo experimentation, together with the transient appearance of the threads make live cell monitoring or additional studies to characterize these structures challenging. Examination by electron microscopy or immunofluorescence microscopy, as well as functional studies using inhibitors and gene knock out models using rodent embryos, may provide further insight. Molecular and gene expression experiments are necessary to determine if there are any associations with known motor proteins such as myosin motors, kinesins and dyneins. This may help determine whether the threads are involved in transporting cellular components from the cytoplasm (Halvaei et al., 2016). The use of live cell imaging, or at least high-resolution and rapid frequency, compared with snap shot, time-lapse image collection, is required to minimize 'inconclusive' categorization and to determine whether thread disassociation occurs after completion of cytokinesis, or if the threads persist throughout the multiple cycles of cell division as observed in the xenopus embryo (Danilchik et al., 2013). The observation of perivitelline threads as late as the blastocyst stage in this study poses the possibility that they are preserved throughout development. Observations of threads in the early embryo and then later in the resulting blastocyst, however, were uncommon (<10% of embryos), but this may warrant further analysis.

Perivitelline threads were confirmed in more than one-half of the embryos examined in varying degrees as noted by the use of three classifications (**Figure 1**). The relationship between the number of threads and the percentage of fragmentation, however, cannot be ascertained owing to the low numbers in each data set.

Nearly one-quarter of embryos did not display threads despite good visualization of the events leading up to first cleavage. The presence of threads did not appear to be correlated with developmental success at later stages, appearing in similar frequencies in both poor- and top-quality embryos, suggesting they are not a predictor of embryo quality. Implantation data from the study is limited, yet among the embryos with known implantation status, implantation rates were marginally higher when threads appeared to be absent in the transferred embryos, compared with when they were identified. Larger cohorts, with live birth data, are needed to determine possible clinical significance. Threads were observed in similar frequencies regardless of ploidy status; however, the number of PGS embryos was small. In addition, two- and three-pronuclei zygotes displayed threads at the same frequency as two-pronuclei zygotes, suggesting threads arise independently of pronuclear status (**Table 2**).

When conventional microscopy is used to examine the PVS of human oocytes, it is common to observe an area where no perivitelline space exists between the cytoplasmic membrane and zona pelluicida, termed a 'cytoplasmic membrane–zona pelluicida contact point'. Only in oocytes with unusually low cytoplasmic volume or in hyperosmotic culture conditions does a space exist around the whole oocyte/zygote. These oocytes are known to result in poor development outcomes (Rienzi et al., 2005; Setti et al., 2011; Zhou et al., 2014).

In most embryos, threads were seen in the PVS at the site specifically associated with prior cytoplasmic membrane–zona pelluicida contact. It remains to be determined if the cytoplasmic membrane actually 'adheres' to zona pellucida at these sites, if the threads are

a consequence of this cytoplasmic membrane–zona pelluicida contact, and whether the threads originate from the plasma membrane, zona pellucida or granulosa cells. In marsupials, an extracellular matrix coat within the inner surface of the zona pellucida has been reported to interact with the cytoplasmic membrane during cleavage (Denker, 2000) but this has not been demonstrated in the human embryo.

The high proportion of threads observed at the first cleavage furrow in an organized array within a discrete area, as opposed to randomly within the perivitelline space, may suggest a possible role of threads in the first cleavage event. Few visual indicators are known to predict the axis of the first cleavage in the human. Polar body position in the PVS has been investigated in the mouse, but is not considered reliable (Hiiragi and Solter, 2004). The maternal spindle has been suggested as an indicator of meridional cleavage in other mammals (Cooke et al., 2003; Hosseini et al., 2016). Microtubular arrays extending from the spindle can be visualized and identified in a non-invasive way by using polarized light (Wang et al., 2001). Similar polarized light experiments visualizing the mitotic spindle and the cytoplasmic membrane–zona pelluicida contact area would be pertinent in investigating any relationship between the spindle, the site of threads from cytoplasmic membrane–zona pelluicida contact area, and cleavage plane.

The association with the cleavage furrow could be coincidental, as a larger PVS is created as the membrane pulls away. Time-lapse videos, however, have shown that perivitelline threads are visible in the PVS of zygotes and not only at the site of the cleavage furrow (**Supplementary Videos 2a** and **2b**). In addition, threads are present as an organized array in a fan-like arrangement (**Supplementary Videos 3a–d** and **Figure 1**) between the zona pellucida and foci within the furrow rather than a random arrangement on the larger surface area of the blastomere membranes or in the entire cleavage furrow.

Embryos that did not display threads at the cleavage furrow or elsewhere implanted after embryo transfer, suggesting that the threads are not necessary for cleavage or normal division. Alternatively, they may be present but are not observed owing to the limitations of time-lapse studies as previously discussed. Pregnancies from zona free (and presumably thread-free) zygotes have been reported (Stanger et al., 2001; Vajta et al., 2010).

Threads were observed to be associated with fragments at the two-cell-stage in most embryos. A causative relationship, however, could not be established here. Only studies using specific staining techniques for these thread structures could determine if this association is real or an artefact of two processes occurring simultaneously. Equally, threads can present at the furrow without the appearance of fragments in a small number of embryos, and the incidence of fragmentation is similar in embryos without threads, which again suggests that this association could be coincidental.

In conclusion, characterization and functional studies are required to further our understanding of the significance of these structures in early embryo development.

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Appendix: Supplementary material

The following is the supplementary data to this article:

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RBMO

ELSEVIER

ARTICLE

Evolution of embryo selection for IVF from subjective morphology assessment to objective time-lapse algorithms improves chance of live birth

BIOGRAPHY

Professor Simon Fishel, Founder and President of CARE Fertility Group and Fellow of the Royal Society of Biology, worked with Nobel Laureate Robert Edwards from 1975 to 1985 at Cambridge University and as Deputy Scientific Director of Bourn Hall. In 1978 he received the Beit Memorial Fellowship and was elected Research Fellow of Churchill College, Cambridge, and in 2009 was awarded an Honorary Fellowship from Liverpool John Moores University for "outstanding contributions to humanity and science in the field of fertility treatment including embryology and IVF".

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KEY MESSAGE

This is the first study to compare an objective time-lapse algorithm (TLIA) of preimplantation embryo development to subjective, conventional blastocyst morphology for embryo transfer and live birth outcome. Detailed analysis of all patient covariates indicates that TLIA was superior for selecting embryos for their propensity to generate a live birth.

ABSTRACT

Research question: Does using an objective time-lapse imaging algorithm (TLIA) after IVF relate to conventional morphological assessment of human blastocysts as a prognosticator for live birth?

Design: Prospective use of a TLIA to select embryos in multicentre IVF clinics all using the same strictly controlled laboratory protocols. Each blastocyst was given a ranking from A to D, with the highest rank preferred for fresh transfer. This ranking was retrospectively compared with a given morphological score, which was blinded to the TLIA rank; all embryos were cultured under the same conditions.

Results: Using multiple variable logistic regression models, TLIA embryo rank enabled greater discrimination between cycles with and without live births than the conventional morphology grade, even when considered in isolation, and when adjusting for covariates related to treatment and patient criteria. Of the 1810 cycles of single blastocyst transfer, 894 (49.4%) resulted in a live birth. A Vuong non-nested test including covariates showed strong evidence of the superiority of the embryo rank model compared with the transfer grade model ($P = 0.0008$ [raw], $P = 0.0003$ [Akaike information criterion – corrected]). From the receiver operating characteristic (ROC) curves across all possible thresholds the TLIA rank showed better true positive and true negative rates and had a higher area under the curve [AUC] of 67.43% compared with 61.74% for the blastocyst morphology grade. The same analysis but excluding covariates demonstrated an AUC of 62.86% versus 54.02%, respectively.

Conclusion: Objective TLIA is superior for selecting embryos for their propensity to generate a live birth over a conventional, subjective blastocyst morphology scoring system.

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INTRODUCTION

Since the introduction of clinical IVF, it is an axiom that not all zygotes have an equal potential for successful development, through the cleavage stages up to live birth, even in the most favourable environment. During the early period of IVF this resulted in the transfer of multiple embryos, and attempts to define viability by morphological criteria (*Edwards et al., 1984*; *Fishel et al. 1983*). Since the routine use of advanced culture systems permitting optimal development to the blastocyst stage, early selection is undertaken to eliminate cleavage-arrested embryos and other anomalies, permitting higher live birth rates with the transfer of single blastocysts (*Gardner et al., 1998*, *2000*; *Marek et al., 1999*; *Van Royen et al., 1999*). Further refinement of blastocyst success rates has been achieved by selecting these embryos using defined morphological criteria (*Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group Embryology, 2011*; *Gardner et al., 2000*). However, it is also acknowledged that morphological criteria alone are a crude and subjective tool in assessing embryo viability (*Hardarson et al., 2012*) because the blastocyst is a dynamic system of changing patterns over short periods of time (*Campbell and Fishel, 2015*). Time-lapse incubators not only provide the unfettered and safe opportunity to observe embryo morphology over its total period of in-vitro culture (*Campbell and Fishel, 2015*) but can also provide objective additional data (algorithms, and observational dysmorphism) that may provide a more accurate assessment of embryo viability (*Campbell et al., 2013a*, *2013c*; *Meseguer et al., 2011*, *2012*; *Pribenszky et al., 2017*; *Rubio et al., 2012*; *Wong et al., 2010*). This study compared the use of objective time-lapse imaging to conventional, subjective morphology criteria for embryo selection.

MATERIALS AND METHODS

The 1810 single embryo transfer cycles were from the CARE Fertility Group clinics at CARE Nottingham, CARE Northampton, CARE Manchester, CARE Sheffield, Beacon CARE Fertility Dublin and CARE London. Patients were self-selected and treatment was based upon appropriate clinical consultation, counselling and decision-making between the patient and treating physician; but all patients decided to include time-lapse imaging in their treatment protocol. Detailed embryology and clinical methodologies and protocols have been previously published (*Fishel et al., 2017*). All embryos were cultured in the same time-lapse incubator system (EmbryoScope; Vitrolife, Sweden), which remained a constant for embryo comparison. Embryos at the blastocyst stage were assessed both using conventional morphological criteria and the in-house derived TLI algorithm. All protocols for patient treatments complied with UK regulations (Human Fertilization and Embryology Act, 1990, 2008) and all UK facilities are regularly inspected by the Human Fertilization and Embryology Authority (HFEA), which includes the use of TLI. The retrospective analysis of the data did not require ethical or institutional review board approval as the clinical and embryology protocols were performed according to previously validated procedures, and practised under licence from the HFEA. All patients were fully counselled and gave their signed, informed consent to their treatment.

Multiple clinical variables were categorized for inclusion in the analysis, and these (with the final groupings applied for each variable) are available as Supplementary Table 1. As well as considering the raw, continuous patient and donor ages in the analysis, patient and donor ages were also grouped as follows: patient age <33, 33–37, ≥38; corresponding to the common grouping used. The groupings for categorizing body mass index (BMI), anti-Müllerian hormone (AMH) and antral follicle count (AFC) were chosen to correspond to clinically meaningful categories, i.e. to reflect what might be considered to be above, below or within a normal/healthy range.

The following definitions were used: (i) BMI: <18.5 (underweight), 18.5–<25 (healthy weight), 25–<30 (overweight), ≥30 (obese); (ii) AMH (pmol/l): <6 (low), 6–<24 (normal), ≥24–<70 (high), 70+ (very high); (iii) AFC: <4 (extremely low), 4–<10 (low), 10–<14 (somewhat low), 14–<22 (normal), 22–<35 (high), 35+ (very high).

The oocyte provider ages (<28, 28–31 and 32+), gonadotrophin dosing days and the total dose were categorized based on the quantiles of the observed distribution to ensure that sufficient information was present in each of the categories for a robust analysis.

Ethnicity was also considered and categorized according to standard ethnic classification into five main groups.

Ovarian stimulation protocols

Pituitary suppression for ovarian stimulation and oocyte maturation has been described previously (*Fishel et al., 2014*).

Oocyte retrieval, denudation and intracytoplasmic sperm injection (ICSI)

Oocyte retrieval was achieved following sedation at approximately 36 h post-human chorionic gonadotrophin injection (10,000 IU; Pregnyl, Organon, UK; or Ovitrelle, Merck Serono) or agonist trigger (buserelin 0.5 ml subcutaneous; Suprefact, Sanofi SA, France); and the cumulus-oocyte complexes were cultured in Ferticult IVF medium (Fertipro, Belgium): all procedures, including IVF and, where appropriate, ICSI, have been described in detail previously (*Fishel et al., 2017*).

Embryo culture and incubation

Details for TLI: the algorithm used, embryo ranking and morphology have been previously published (*Fishel et al., 2017*). The morphology scoring system, which was not considered by the algorithm, was modified from the Gardner and Schoolcraft AA/AB/BA, etc. annotation of blastocyst trophectoderm and inner cell mass morphology (*Gardner and Schoolcraft, 1999*) and was replaced by numbers: 1:1/1:2/2:1, etc. as per the Istanbul consensus recommendation (*Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group Embryology, 2011*). The switch to numeric was to distinguish from the alphabet-based algorithm ranking.

Evaluation of time-lapse images, annotation protocols and quality control

The evaluation and use of TLI in the study system, the timing allowance for ICSI, annotation protocols and the strict training and adherence criteria for quality control have all been detailed previously (*Fishel et al., 2017*, *2018*). The algorithm did not include morphological criteria or cleavage dysmorphism, and although

extensively validated, the effectiveness of an in-house derived algorithm may not produce similar results in other settings (*Freour et al., 2015*; *Liu et al., 2016*; *Storr et al., 2018*), as previously discussed (*Fishel et al., 2018*).

Embryo selection and transfer

Strict user-defined time-lapse algorithms as published previously (*Fishel et al., 2017*) were used for objective selection of embryos. Briefly, for blastocysts, these were preferentially selected with a tSB (time to the start of blastulation in hours after insemination) ≤93.1. If this criterion was not met, preference was given to embryos where the duration of blastulation (dB) = the time to blastulation – from ICSI or insemination [tB] minus time to the start of blastulation [tSB]) was ≤12.5 h. The decision to transfer a particular embryo was made following annotation, with A-ranked embryos given the highest priority and a rank of D the lowest. Morphology grade, which was not used for embryo selection, was scored by an experienced embryologist at the time of transfer, but blinded to the embryo rank by the TLIA. Embryo transfer was performed under ultrasound guidance using one of several catheters (outlined in Supplementary Table 1).

Statistical methods

Separate multiple variable logistic regression models were fit to the study data to assess the discriminatory performance of embryo rank and transfer grade on live births. A logistic regression model computes the probability of the binary outcome as a function of the supplied explanatory variables (*Agresti, 2013*; *Hastie et al., 2009*; *Pohar Perme et al., 2004*; *Sperandei, 2014*). Other covariates, aside from embryo rank and transfer grade, respectively, were considered for inclusion in the models to control for potential confounding. These covariates aim explicitly to control for differences in the patient populations between the embryo rank groups and the transfer grades, respectively, in the two separate models. Morphology assessment information (i.e. transfer grade) was not considered as a potential confounder in the embryo rank model and vice versa.

The variables made available to the variable selection procedure and in the final models for embryo rank and transfer grade, respectively, are given in Supplementary Table 1.

The available interactions were limited to the following:

- Patient age with an indicator for whether the patient was an oocyte recipient, to allow for the possibility that the patient's age is less (or even more) important when they have received an egg donation.
- Embryo rank/transfer grade with each of patient age, oocyte recipient indicator, donor age (where applicable), day of embryo transfer, ICSI and array comparative genomic hybridization. These interactions were included to allow for the possibility that the sizes and directions of the embryo rank/transfer grade effects vary for different groups of patients.
- A three-way interaction between embryo rank/transfer grade, oocyte recipient status and patient age. This was included because the corresponding two-way interaction without embryo rank/transfer grade was being included. Consideration was also given to the inclusion of higher-order effects of patient age, i.e. quadratic age terms were considered to allow for a non-linear effect of age (on the log-odds of live births). Where evidence of a quadratic age term was found, interactions with this term were also considered, consistent with the interactions considered for the main effect of age.

The models were also run with no covariates to explore the predictive performance of embryo rank and transfer grade in isolation. These models, without covariates, demonstrate how well each assessment predicts the live births outcome without taking account of any other information about the cycles.

ROC curves were compared between embryo rank and transfer grade, and the AUC values achieved by the embryo rank and transfer grade models; these represent the accuracy of the predictive model, where 1 is the best possible value, and 0.5 is equivalent to predicting at random.

The fitted and final models were also used to provide estimates of the directions and sizes of the effects of interest (presented as odds ratios [OR]), and the estimated effect sizes are accompanied by confidence intervals (CI). Profile likelihood CI are provided, which are more reliable than the more commonly presented Wald intervals (i.e. estimate ± percentile × SE of estimate). Likelihood ratio tests (LRT) were also calculated to assess the overall significance of each variable, across the multiple groups of a categorical variable (where applicable).

All analyses were performed in the statistical software package R version 3.4.3 (2017-11-30) (R Core Team, 2017). The ModelGood package was used to implement the ROC curves (and the pscl package was used to apply Vuong's test; *Jackman, 2017*).

The variables made available to the variable selection procedure and those that were selected to be in the final models for embryo rank and transfer grade, respectively, are not presented but can be accessed in the Supplementary Materials, along with descriptive statistics used to characterize the covariates available across the embryo ranks and transfer grades.

RESULTS

There were 1810 cycles for analysis, 1373 cycles of standard (own eggs) patients, and 437 cycles of oocyte donation. Of these 996 (996/1810; 55.0%) had an embryo rank of A, 568 (568/1810; 31.4%) of B, 208 (208/1810; 11.5%) of C, and 38 (38/1810; 2.1%) of D. Whereas 206 (206/1810; 11.4%) had a transfer grade of 1:1, 223 (223/1810; 12.3%) had 1:2, 77 (77/1810; 4.3%) had 2:1, 1095 (1095/1810; 60.5%) had 2:2, 149 (149/1810; 8.2%) had 2:3, 28 (28/1810; 1.5%) had 3:2, and 32 (32/1810; 1.8%) had 3:3. Of the 1810 cycles, 894 (49.4%) resulted in live births. These were: 589 (59.1%) for transfers with an embryo rank of A, 253 (44.5%) for B, 50 (24%) for C and 2 (5.3%) for D. In terms of transfer grades: 110 (53.4%) for transfers with a transfer grade of 1:1, 114 (51.1%) for 1:2, 43 (55.8%) for 2:1, 568 (51.9%) for 2:2, 47 (31.5%) for 2:3, 5 (17.9%) for 3:2 and 7 (21.9%) for 3:3.

All patients

Supplementary Table 1 shows that the variables selected to be included in the final models for embryo rank were embryo rank, patient age, low molecular weight heparin (Clexane®), patient type (standard/oocyte recipient), gonadotrophin dosing days, embryo rank by patient age interaction, embryo rank by day of transfer interaction, and embryo rank by ICSI interaction; and

TABLE 1 ESTIMATED COEFFICIENTS AND OR (95% CI) FOR THE LIVE BIRTHS OUTCOME EMBRYO RANK MODEL

Variable	Comparison	Coefficient	SE	OR	95% CI	z-statistic	Wald *P*-value	LRT *P*-value
–	(Intercept)	4.3122	0.708	74.6037	[18.947, 304.259]	6.09	<0.0001	–
Embryo rank	Rank B versus A	–3.7174	0.945	0.0243	[0.004, 0.155]	–3.93	0.0001	<0.0001
Embryo rank	Rank C versus A	–4.4926	1.326	0.0112	[0.001, 0.152]	–3.39	0.0007	<0.0001
Embryo rank	Rank D versus A	–11.3419	5.807	<0.0001	[<0.0001, 0.255]	–1.95	0.0508	<0.0001
Embryo rank / day of transfer	A: 5–7 versus 2–4	–1.0443	0.294	0.3519	[0.192, 0.611]	-3.55	0.0004	0.0009
Embryo rank / day of transfer	B: 5–7 versus 2–4	–0.4146	0.382	0.6606	[0.307, 1.391]	–1.09	0.2775	0.0009
Embryo rank / day of transfer	C/D: 5–7 versus 2–4	0.3101	0.401	1.3636	[0.641, 3.122]	0.77	0.4387	0.0009
Embryo rank / ICSI	A: Yes versus No	–0.5418	0.154	0.5817	[0.429, 0.785]	–3.52	0.0004	0.0045
Embryo rank / ICSI	B: Yes versus No	0.0843	0.178	1.0879	[0.767, 1.544]	0.47	0.6363	0.0045
Embryo rank / ICSI	C/D: Yes versus No	–0.0912	0.336	0.9128	[0.476, 1.784]	–0.27	0.7858	0.0045
Patient age (years)	Per year: A	–0.0973	0.016	0.9073	[0.879, 0.936]	–6.09	<0.0001	<0.0001
Patient age (years) / embryo rank	Per year: B versus A	0.0599	0.022	1.0618	[1.017, 1.109]	2.72	0.0065	0.0308
Patient age (years) / embryo rank	Per year: C versus A	0.0400	0.034	1.0408	[0.972, 1.112]	1.17	0.2413	0.0308
Patient age (years) / embryo rank	Per year: D versus A	0.1779	0.147	1.1947	[0.908, 1.689]	1.21	0.2264	0.0308
Clexane	Yes versus No	–0.2447	0.143	0.7829	[0.591, 1.036]	–1.71	0.0872	0.0865
Patient type	Oocyte recipient versus standard	1.2572	0.299	3.5155	[1.965, 6.349]	4.21	<0.0001	<0.0001
Gonadotrophin dosing days	Per day	0.0783	0.024	1.0815	[1.031, 1.135]	3.21	0.0013	0.0012

z-statistic is the test statistic for a Wald test of the significance of the OR for the corresponding comparison. The LRT tests the significance of the variable across the multiple categories, where applicable.

CI = confidence interval; ICSI = intracytoplasmic sperm injection; LRT = likelihood ratio test; OR = odds ratio; SE = standard error.

those included for transfer grade were transfer grade, patient age, total previous cycles, Clexane®, patient type (standard/oocyte recipient), gonadotrophin dosing days, patient type by patient age interaction and transfer grade by ICSI interaction. Having accounted for these variables, there was no evidence of the other, omitted covariates being associated with the odds of a live birth.

The embryo rank model results (TABLE 1) provide strong evidence of an effect of embryo rank (LRT *P*-value <0.0001), presented as OR relative to a rank of A, which are the ratios of the odds of a live birth with an embryo of rank B, C or D to the odds of a live birth with an embryo of rank A. For example, the estimated OR for an embryo of rank C compared with A is 0.0112 with a 95% CI of 0.001–0.152, indicating that there is strong evidence (Wald *P*-value 0.0007) that embryos of rank C are less likely to result in a live birth than embryos of rank A.

The estimated OR associated with the other covariates retained in the final embryo rank model are also presented in TABLE 1. The data suggest, for example, that the odds of a live birth are higher for oocyte recipients compared with standard patients (OR 3.5155; 95% CI 1.965–6.349).

As the model contains both the main effect of embryo rank as well as interactions between embryo rank and other variables, the LRT for the embryo rank variable presented in Supplementary Table 14 considers the effect of omitting the embryo rank variable having already dropped the embryo rank/day of transfer interaction, embryo rank/patient age and embryo rank/ICSI terms from the model.

The transfer grade model results are given in TABLE 2, which provide strong evidence of an effect of transfer grade (LRT *P*-value <0.0001). The effect sizes are presented as OR relative to a grade of 1:1, which are the ratios of the odds of a live birth with an embryo of rank 1:2, 2:1, 2:2, etc. to the odds of a live birth with an embryo of grade 1:1. For example, the estimated OR for an embryo of grade 3:2 compared with 1:1 is 0.2757 with a 95% CI of 0.081–0.811, indicating that there is evidence (Wald *P*-value 0.0261) that embryos of grade 3:2 are less likely to result in a live birth than embryos of grade 1:1. As the model includes both quadratic and interaction terms with age, the LRT for the associated terms consider omitting the individual effects having already removed any higher-order terms with age. Similarly, the LRT for transfer grade considers the effect of omitting transfer grade having already dropped the transfer grade/ICSI interaction terms from the model.

A Vuong non-nested test of the final models showed strong evidence of the superiority of the embryo rank model (including covariates) compared with the transfer grade model (including covariates) ($P = 0.0008$ [raw], $P = 0.0003$ [AIC-corrected]). FIGURE 1 shows the ROC curves for the final models. Across all possible thresholds, the embryo rank model showed better true positive and true negative rates and had a higher AUC of 67.43% compared with 61.74% for the transfer grade model, indicating a superior predictive accuracy for embryo selection by the TLI algorithm.

Excluding all covariates, similar results were obtained although a weaker performance overall. A Vuong non-nested test of the models without covariates showed strong evidence of the superiority of the embryo rank model compared with the transfer grade model

TABLE 2 ESTIMATED COEFFICIENTS AND OR (95% CI) FOR THE LIVE BIRTHS OUTCOME TRANSFER GRADE MODEL

Variable	Comparison	Coefficient	SE	OR	95% CI	z-statistic	Wald *P*-value	LRT *P*-value
–	(Intercept)	–7.4540	3.001	0.0006	[0.000, 0.201]	–2.48	0.0130	–
Total previous cycles	Per cycle	–0.0609	0.049	0.9410	[0.854, 1.035]	–1.24	0.2155	0.2136
Clexane®	Yes versus No	–0.2636	0.146	0.7682	[0.576, 1.023]	–1.80	0.0717	0.0713
Patient type	Oocyte recipient versus standard	7.9506	5.879	>1000	[0.032, >1000]	1.35	0.1762	0.1716
Transfer grade	1:2 versus 1:1	–0.1627	0.312	0.8499	[0.460, 1.569]	–0.52	0.6024	<0.0001
Transfer grade	2:1 versus 1:1	0.0373	0.448	1.0381	[0.435, 2.551]	0.08	0.9335	<0.0001
Transfer grade	2:2 versus 1:1	–0.4512	0.239	0.6368	[0.397, 1.014]	–1.89	0.0588	<0.0001
Transfer grade	2:3 versus 1:1	–0.4798	0.384	0.6189	[0.289, 1.312]	–1.25	0.2118	<0.0001
Transfer grade	3:2 versus 1:1	–1.2886	0.579	0.2757	[0.081, 0.811]	–2.22	0.0261	<0.0001
Transfer grade	3:3 versus 1:1	–0.9500	0.561	0.3867	[0.123, 1.131]	–1.69	0.0903	<0.0001
Transfer grade / ICSI	1:1: Yes versus No	–0.3666	0.289	0.6931	[0.392, 1.220]	–1.27	0.2049	0.0463
Transfer grade / ICSI	1:2: Yes versus No	–0.2604	0.289	0.7707	[0.436, 1.356]	–0.90	0.3677	0.0463
Transfer grade / ICSI	2:1: Yes versus No	–0.4667	0.493	0.6271	[0.234, 1.638]	–0.95	0.3442	0.0463
Transfer grade / ICSI	2:2: Yes versus No	0.1358	0.136	1.1455	[0.878, 1.495]	1.00	0.3176	0.0463
Transfer grade / ICSI	2:3/3:2/3:3: Yes versus No	–0.9570	0.361	0.3840	[0.189, 0.782]	–2.65	0.0080	0.0463
Gonadotrophin dosing days	Per day	0.0846	0.025	1.0883	[1.037, 1.143]	3.42	0.0006	0.0006
Patient age (years)	Per year: standard	0.5244	0.181	1.6894	[1.188, 2.421]	2.90	0.0037	<0.0001
Patient age (years) / patient type	Per year: oocyte recipient versus standard	–0.4733	0.314	0.6230	[0.332, 1.145]	–1.51	0.1314	0.1151
Patient age (years) – quadratic	Per year^2: standard	–0.0092	0.003	0.9908	[0.985, 0.996]	–3.38	0.0007	0.0041
Patient age (years) – quadratic / patient type	Per year^2: oocyte recipient versus standard	0.0080	0.004	1.0080	[1.000, 1.017]	1.89	0.0582	0.0561

z-statistic is the test statistic for a Wald test of the significance of the OR for the corresponding comparison. The LRT tests the significance of the variable across the multiple categories, where applicable.

CI = confidence interval; ICSI = intracytoplasmic sperm injection; LRT = likelihood ratio test; OR = odds ratio; SE = standard error.

($P < 0.0001$). FIGURE 2 shows the ROC curves for the models without covariates. Across all possible thresholds, the embryo rank model showed better true positive and true negative rates and had a higher AUC of 62.86% compared with 54.02% for the transfer grade model.

Standard patients (non-oocyte recipient) only

Of the 1373 cycles for standard patients included in the analysis, 679 (49.5%) resulted in live births. These were 441 (60.9%) for transfers with an embryo rank of A, 197 (43.4%) for B, 39 (24.1%) for C and 2 (6.1%) for D. In terms of transfer grades: 78 (49.4%) for transfers with a transfer grade of 1:1, 83 (50.6%) for 1:2, 30 (60%) for 2:1, 446 (53.3%) for 2:2, 33 (28.9%) for 2:3, 3 (15%) for 3:2 and 6 (20%) for 3:3.

The embryo rank model results are given in Supplementary Table 14, which provide strong evidence of an effect of embryo rank (LRT *P*-value <0.0001). Again, the effect sizes are presented as OR relative to a rank of A, which are the ratios of the odds of a live birth with an embryo of rank B, C or D to the odds of a live birth with an embryo of rank A. For example, the estimated OR for an embryo of rank C compared with A is 0.0314 with a 95% CI of 0.008–0.107, indicating that there is strong evidence (Wald *P*-value <0.0001) that embryos of rank C are less likely to result in a live birth than embryos of rank A. As the model contains both the main effect of embryo rank as well as interactions with this variable, the LRT for the embryo rank variable (presented in Supplementary Table 14) considers the effect of omitting the embryo rank variable having already dropped the embryo rank/day of transfer interaction and embryo rank/ICSI terms from the model.

The transfer grade model results are given in Supplementary Table 15, which provide strong evidence of an effect of transfer grade (LRT *P*-value <0.0001). The effect sizes are again presented as OR relative to a grade of 1:1, which are the ratios of the odds of a live birth with an embryo of rank 1:2, 2:1, 2:2, etc. to the odds of a live birth with an embryo of grade 1:1. For example, the estimated OR for an embryo of grade 3:2 compared with 1:1 is 0.1619 with a 95% CI of 0.036–0.520, indicating that there is strong evidence (Wald *P*-value 0.0056) that embryos of grade 3:2 are less likely to result in a live birth than embryos of grade 1:1.

A Vuong non-nested test of the final models for standard patients showed strong evidence of the superiority of the embryo rank model (including covariates) compared with the transfer grade model (including covariates) ($P = 0.0005$). The ROC curves for the final models for standard patients only are provided in Supplementary Figure 1. Across all possible thresholds, the embryo rank model showed better true positive and true negative rates and had a higher AUC

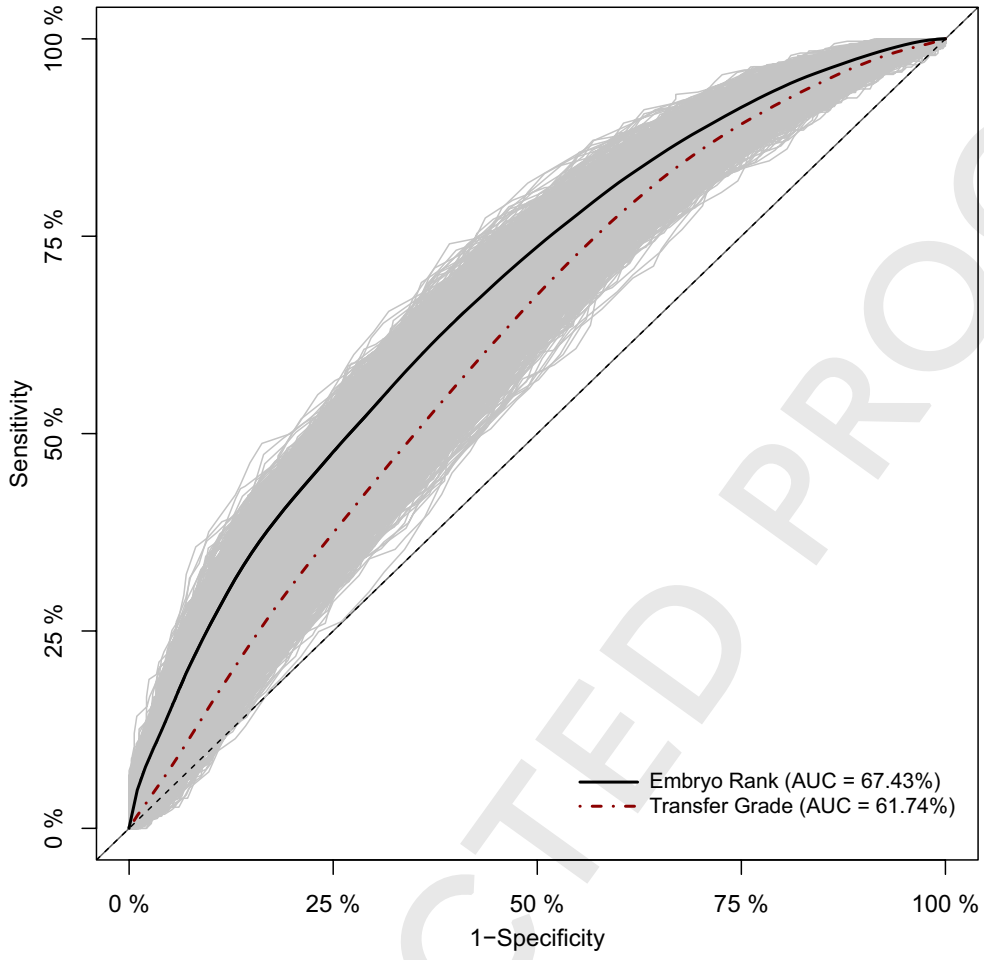

FIGURE 1 Receiver operating characteristic (ROC) curves (and associated areas under the curve [AUC]) showing the discriminative performance between cycles with and without live births for the embryo rank and transfer grade predictive models (including covariates) for all patients. Generalization error is estimated via internal bootstrap cross-validation with the grey lines showing the out-of-sample performance estimated across 1000 bootstrap samples and the black and red lines the average estimates, respectively.

of 68.86% compared with 63.73% for the transfer grade model.

Excluding all covariates, similar but overall weaker performing results were obtained. A Vuong non-nested test of the models without covariates for standard patients showed strong evidence of the superiority of the embryo rank model compared with the transfer grade model (P = 0.0016). The ROC curves for the models for standard patients without covariates are shown in Supplementary Figure 2. Across all possible thresholds, the embryo rank model showed better true positive and true negative rates and had a higher AUC of 64.32% compared with 55.52% for the transfer grade model.

Oocyte recipients

Of the 437 oocyte recipient cycles, 215 (49.2%) resulted in live births. These were: 148 (54.4%) for transfers with an embryo rank of A, 56 (49.1%) for B, 11 (23.9%) for C and 0 for D. In terms of transfer grades: 32 (66.7%) for transfers with a transfer grade of 1:1, 31 (52.5%) for 1:2, 13 (48.1%) for 2:1, 122 (47.3%) for 2:2, 14 (40%) for 2:3, 2 (25%) for 3:2 and 1 (50%) for 33.

Excluding all covariates, a Vuong non-nested test of the models for oocyte recipient patients showed weak evidence of the superiority of the embryo rank model compared with the transfer grade model when corrected for the number of coefficients included in the models (P = 0.1459). The ROC curves for the models for oocyte recipient patients without covariates are shown in Supplementary Figure 3. At the lower probability thresholds (towards the right-hand side of the figure), the embryo rank model showed better true positive and true negative rates and had a higher overall AUC of 56.70% compared with 53.13% for the transfer grade model.

DISCUSSION

This is the first study to compare an objective TLIA to conventional, subjective morphology assessment, and using live birth as the endpoint incorporating a comprehensive array of potential clinical confounders. Comprehensive statistical analysis reveals the superior selection power of the TLIA over the conventional blastocyst morphology scoring system (*Gardner et al., 2000*). However, the study confirmed that the Gardner blastocyst morphology assessment is in itself a useful discriminator for live birth potential – even though weaker than the TLIA model.
An earlier study (*Armstrong et al., 2015*) on TLIA ranking of embryos for their propensity for live birth also indicated the value of conventional morphology for live birth assessment, but highlighted the variability and flawed reliance solely on the top-ranking grades. In a more recent study on ranking embryos by TLIA (*Fishel et al.,*

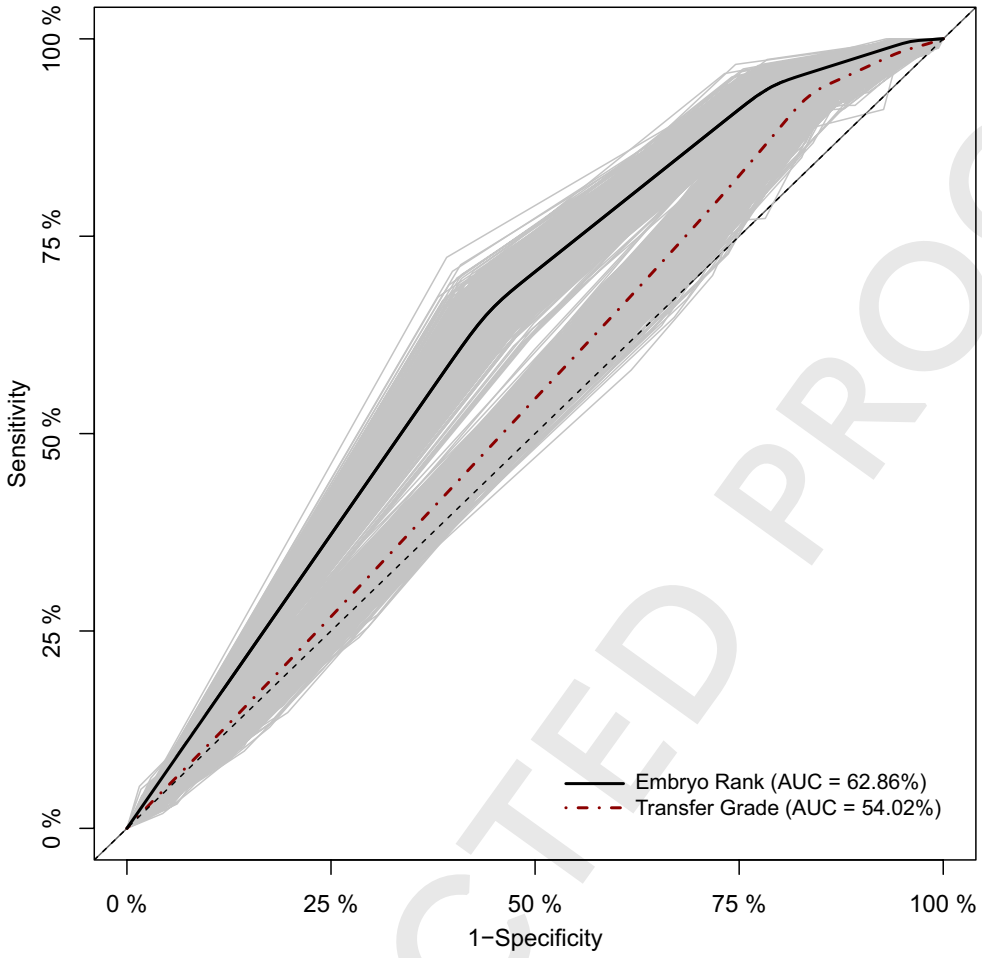

FIGURE 2 ROC curves (and associated AUC) showing the discriminative performance between cycles with and without live births for the embryo rank and transfer grade predictive models (excluding covariates) for all patients. Generalization error is estimated via internal bootstrap cross-validation with the grey lines showing the out-of-sample performance estimated across 1000 bootstrap samples and the black and grey lines the average estimates, respectively.

2018) blastocyst grade 2:2 morphology appeared to have the highest selective value. Embryos, especially blastocysts, are known to have highly dynamic, morphologically changing profiles that may vary within a short time-frame, but up to now single microscopic assessment has been the best system available. Selecting an embryo on its morphological appearance at a single, short time point in a 24 h period gives only a snapshot that may change from, for example, a 2:2 to a 1:1 score, within an hour of assessment, notwithstanding the subjective view of the embryologist at all times. Time-lapse imaging, even without an algorithm based on morphokinetics, simply using video toggling through its morphological vicissitudes, demonstrates this temporal variability and, hence, the vagary of clinical dependence.

The data in this study provide evidence of time-lapse imaging embryo rank enabling greater discrimination between cycles with and without live births compared with using conventional transfer grade (morphology) assessment. This is evident when the predictive performance of embryo rank and transfer grade are considered in isolation, but more importantly, when adjusting for available covariates related to treatment and patient histories.

Patient type, i.e. standard or oocyte recipients, was controlled for in the analysis adjusting for covariates; this has been an important omission in some recent publications and one of the drawbacks to acquiring any positive support for TLIA in a recent Cochrane Review (*Armstrong et al., 2018*). As an additional sensitivity analysis, the assessments were also compared across cycles stratified by patient type (standard or oocyte recipient patients). The results for both standard and oocyte recipient patients were consistent with the main analysis, i.e. embryo rank provided greater discrimination between cycles with and without live births than embryo morphology assessment.

Since IVF gained acceptance as a clinical service in the 1980s, and with the introduction of ovarian stimulation and the resultant multiple embryos, finding discriminators of embryo viability became more important (*Brison et al., 2004*; *Edwards et al., 1984*; *Fishel et al. 1983*; *Fragouli et al., 2010*; *Katz-Jaffe et al., 2009*; *Seli et al., 2008*). But attempts to ensure the highest chance of a pregnancy resulted (and in many clinics still does) in the transfer of multiple embryos with the negative consequence of high-order multiple pregnancies. Since the 1980s it has been appreciated that not all embryos have the same potential for live birth; indeed, a potential high incidence of

aneuploidy was first recognized by *Angel et al. (1986,1983)* and it was well established that the implantation and live births were materially lower than the number of embryos transferred to the same uterus. Great strides have been made over the last decade to maintain or even improve live birth rates by using only a single blastocyst transfer and various selection tools. However, still the most widely used selection protocol is a subjective, single point morphological assessment of the embryo before transfer. More recently, the introduction of time-lapse imaging has been importantly instructive; objectively demonstrating a high incidence of morphological dysmorphism during cleavage, concomitant with reduced implantation and live births, providing sophisticated morphokinetic computations as a prognosticator for embryo viability (*Campbell and Fishel, 2015*; *Lagalla et al., 2016*; *Pribenszky et al., 2017*; *Reignier et al., 2018*; *Viñals Gonzalez et al., 2018*; *Zhan et al., 2016*), and introducing machine learning to evolve independent selective algorithms for automated programming (*Kohsravi et al., 2019*; *Tran et al., 2019*).

The future for IVF is to continue to improve live birth rates while eliminating the risk of multiple pregnancy, and while all protocols and technical procedures can be optimized the immutable fact of human procreation – that only a proportion of embryos have the capacity to make a baby, however conceived – will ensure the continued drive in IVF clinics to discover and automate powerful tools for predicting embryo viability. This study not only indicates that TLIA is an appropriate step in that direction, it is also indicative that TLIA is unlikely to be binary, as would be an accurate assessment of ploidy, for example; hence TLIA will rarely be used to rule out embryo transfer or reduce unnecessary cryopreservation of embryos, as would the knowledge that the embryo is aneuploid, but will act as an objective hierarchical ranking system that should reduce the time to live birth. Several studies have tried to link TLIA to embryo ploidy, with mixed uncertain correlation (*Campbell et al., 2013b*; *Minasi et al., 2016*; *Rienzi et al., 2015*; *Zhang et al., 2017*), and although it may show cleavage anomaly in gross aneuploid embryos it is unlikely to be successful in discriminating subtle, but devastating, single trisomy.

Some of the OR estimated in this study have rather wide CI that contain 1, which means in these cases a strong conclusion cannot be reached about the size or direction of the effect in question. Collecting more data to increase the sample size would tend to reduce the width of the CI, so may help to establish whether the estimated effect is real.

By including in the models all the covariates that are available (and that the data supports), an attempt has been made to account for imbalances between the embryo rank groups or transfer grade groups. However, it is not possible to account for any differences not contained within the available data, so it is also impossible to guarantee that all confounding effects have been eliminated (e.g. factors that predispose a patient to both having a particular embryo rank or transfer grade and to having a successful outcome). As such, this study cannot be used to draw strong conclusions about causal links between embryo rank or transfer grade and birth outcomes. In general, a randomized controlled study is required in order to draw strong conclusions about causality, but the undertaking of a prospective, randomized controlled trial using morphology or TLIA as the only selection option in enough patients that can be controlled for all clinical confounders will remain a challenge.

UNCITED REFERENCES

(*Franasiak et al., 2014*, *R Core Team 2016*, *Tran et al., 2018*, *The Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting 2014*)

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SF, AB and AC prepared and assessed the data, and statistical analysis was undertaken by an independent, external, professional organization.

ETHICAL APPROVAL

This work is covered by licence from the Human Fertilisation and Embryology Authority (HFEA). Patients provide informed consent, and all such consents are inspected by the HFEA. As per the Ethics Committee review of this ongoing work in relation to the previously published manuscript in Rbmonline in 2018 (*"Time-lapse imaging algorithms rank human preimplantation embryos according to the probability of live birth: RBMO VOLUME 37 ISSUE 3 2018"*), (see attached Ethics Committee letter dated 2017) and that it involves only database interrogation it did not require further ethics committee approval.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.rbmo.2019.10.005.

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RBMO

ARTICLE

Time-lapse imaging algorithms rank human preimplantation embryos according to the probability of live birth

BIOGRAPHY

Simon Fishel, Founder and President of CARE Fertility Group, Fellow of the Royal Society of Biology worked with IVF pioneer and Nobel Laureate Robert Edwards from 1975-1985 at Cambridge University and as Deputy Scientific Director of the first IVF clinic, Bourn Hall. In 1978 he received the Beit Memorial Fellowship and was elected Research Fellow of Churchill College, Cambridge. In 2009 was awarded Liverpool John Moores University Honorary Fellowship for "outstanding contributions to humanity and science in the field of fertility treatment including embryology and IVF"

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KEY MESSAGE

This retrospective study demonstrated for the first time that human blastocyst embryos can be objectively ranked according to their propensity to produce a live birth using an in-house derived morphokinetic-based algorithm from time-lapse imaging. This appears to have greater discriminating power than subjective, conventional morphology assessment.

ABSTRACT

Research question: Can blastocysts leading to live births be ranked according to morphokinetic-based algorithms?

Design: Retrospective analysis of 781 single blastocyst embryo transfers, including all patient clinical factors that might be potential confounders for the primary outcome measure of live birth, was weighed using separate multi-variable logistic regression models.

Results: There was strong evidence of effect of embryo rank on odds of live birth. Embryos were classified A, B, C or D according to calculated variables; time to start (tSB) and duration (dB{tB – tSB}) of blastulation. Embryos of rank D were less likely to result in live birth than embryos of rank A (odds ratio [OR] 0.3046; 95% confidence interval [CI] 0.129, 0.660; $P < 0.005$). Embryos ranked B were less likely to result in live birth than those ranked A (OR 0.7114; 95% CI 0.505, 1.001; $P < 0.01$), and embryos ranked C were less likely to result in live birth than those ranked A (OR 0.6501, 95% CI 0.373, 1.118; $P < 0.01$). Overall, the LRT (Likelihood Ratio Test) p-value for embryo rank shows that there is strong evidence that embryo rank is informative as a whole in discriminating between live birth and no live birth outcomes ($p = 0.0101$). The incidence of live birth was 52.5% from rank A, 39.2% from rank B, 31.4% from rank C and 13.2% from rank D.

Conclusions: Time-lapse imaging morphokinetic-based algorithms for blastocysts can provide objective hierarchical ranking of embryos for predicting live birth and may have greater discriminating power than conventional blastocyst morphology assessment.

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KEYWORDS

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Selection algorithm
Time lapse

INTRODUCTION

Assessment of the value of time-lapse imaging (TLI) following its recent introduction into clinical IVF practice (*Cruz et al., 2011; Pribenszky et al., 2010; Wong et al., 2010*) has largely centred on the incidence of pregnancy in comparison to conventional culture (*Rubio et al., 2014; Wu et al. 2016*). Some studies have tried to evaluate algorithms predictive of blastulation (*Cruz et al., 2012; Dal Canto et al., 2012; Hashimoto et al., 2016; Herrero et al., 2013; Kirkegaard et al., 2014; Milewski et al., 2015; Motato et al., 2016*), and others have searched for algorithms predictive of euploidy or aneuploidy (*Campbell et al., 2013a,b; Del Carmen et al., 2017; Franasiak et al., 2014; Kramer et al., 2014; Lagalla et al., 2017; Minasi et al., 2016; Mumusoglu et al., 2017; Rienzi et al., 2015*). Of late, there have been several reviews looking at the use of TLI in all these domains (*Armstrong et al., 2015; Milewski and Ajduk, 2017; Polanski et al., 2014; Pribenzsky et al., 2017; Racowsky et al. 2015*). A large retrospective analysis of live births comparing TLI to conventional culture was recently published, concluding that the former can improve the incidence of live births by 19% in this system (*Fishel et al., 2017*). There are conflicting views on the value of TLI for improving IVF outcome, which in part is due to what has been measured; for instance, assessing TLI outcome solely while treating the embryo as an independent factor (see *Kirkegaard et al., 2016*); or comparing TLI algorithms to using a time-lapse incubator as a closed incubation system only, without considering any algorithms (*Rubio et al., 2014*). Furthermore, different days of embryo transfer and different culture systems (*Ciray et al., 2012*), and different embryo phenotypes (*Athayde Wirka et al., 2014*) have been used. Few studies have focused on live birth outcome.

In this retrospective analysis, treatment outcome using single embryo transfer at the blastocyst stage was examined; all embryos were cultured in the same TLI device with an identical culture system. All potentially confounding clinical factors were evaluated, and an assessment was done of whether embryos could be objectively and successfully ranked for their potential to result in a live birth based on a simple TLI algorithm. The relevance of conventional blastocyst morphology in comparison to using the TLI algorithm was also tested.

MATERIALS AND METHODS

This multicentre study included 843 transfers for 781 unique patients attending CARE fertility centres from January 2013 to December 2015, at CARE Nottingham, CARE Northampton, CARE Manchester, CARE Sheffield, Beacon CARE Fertility Dublin and CARE London. All embryos were cultured in the EmbryoScope (Vitrolife, Sweden). Only embryos at the blastocyst stage were assessed, both using conventional morphological criteria and the TLI algorithm. All protocols for patient treatments complied with UK regulation (Human Fertilization and Embryology Act, 1990, 2008) and all UK facilities are regularly inspected by the Human Fertilization and Embryology Authority (HFEA), which includes the use of TLI. The retrospective analysis of the use of TLI algorithms for embryo selection did not require ethical or Institutional Review Board (IRB) approval, as confirmed by the chair of the IRB on 13 January 2017, having been performed according to previously validated procedures, and practised under licence from the HFEA. All patients were fully counselled and gave their signed consent. TLI was undertaken using the EmbryoScope with strict adherence to annotation protocols. All embryos were selected for transfer based on their in-house-derived TLI algorithm rank for transfer; standard morphology of the selected embryos was also recorded in the conventional manner at embryo transfer. The primary end-point of this study was a live birth event, i.e. the number of patients achieving a delivery of a live birth for each embryo transfer. Only 'fresh' single embryo transfer cases were included, and all preimplantation genetic testing cases were excluded.

The following clinical variables were categorized for inclusion in the analysis: patient age, day of embryo transfer, number of embryos transferred, donor age (where applicable), body mass index (BMI), anti-Müllerian hormone (AMH), antral follicle count (AFC), gonadotrophin type, gonadotrophin dosing days and gonadotrophin total dose. The groupings applied are presented for each variable in TABLE 1. Patient age was considered as a binary variable in the modelling (<38 and 38+), corresponding to the common grouping used by HFEA, which is familiar to patients. The groupings for categorizing BMI, AMH and AFC were chosen to correspond with clinically meaningful categories, i.e. to reflect what might be considered to be above, below or within a normal/healthy range. The oocyte provider ages (<29, 29–32 and 33+), gonadotrophin dosing days and the total dose were categorized based on the quantiles of the observed distribution to ensure that sufficient information was present in each of the categories for a robust analysis.

The following definitions were used for the BMI, AMH and AFC categories, relating to the data presented:

- BMI: <18.5 (underweight), 18.5–<25 (healthy weight), 25–<30 (overweight), 30–<40 (obese), 40+ (extremely obese).
- AMH (pmol/l): <6 (low), 6–<24 (normal), 24–<70 (high), 70+ (very high).
- AFC: <4 (extremely low), 4–<10 (low), 10–<14 (somewhat low), 14–<22 (normal), 22–<35 (high), 35+ (very high).

OVARIAN STIMULATION PROTOCOLS

Pituitary suppression for ovarian stimulation was performed either with gonadotrophin-releasing hormone agonist (Suprecur; 0.5 ml subcutaneously daily; Sanofi Aventis, UK) or antagonist (Cetrotide; 0.25 mg daily; Merck Serono, UK), and ovarian stimulation was achieved using human menopausal gonadotrophin (Menopur; Ferring, UK) and/or recombinant FSH (Gonal-F; Merck Serono), as previously described (*Campbell et al., 2013b; Fishel et al., 2016*).

TABLE 1 VARIABLES OFFERED TO THE MODELS DURING THE STEPWISE SELECTION PROCEDURE. FOR EACH OUTCOME RESPECTIVELY, THOSE MARKED Y WERE INCLUDED IN THE FINAL MODEL

Variable	Included in live births model?	Included in implantation model?	Included in clinical miscarriage model?
Patient age (years)	Y	Y	Y
Embryo rank (A/B/C/D)	Y	Y	N
Total previous cycles	Y	Y	Y
Total previous miscarriages	N	N	Y
Aspirin (Y/N)	N	N	N
Intralipids (Y/N)	N	N	N
Clexane (Y/N)	N	N	N
Prednisolone (Y/N)	N	N	N
BMI	N	N	N
Day 2 FSH	N	N	N
AMH	N	N	N
AFC	N	N	N
Gonadotrophin type	Y	Y	N
Maximum endometrial thickness (mm)	N	Y	N
Ethnicity	N	N	N
Duration of infertility (years)	N	N	N
Gonadotrophin total dose	N	N	N
Gonadotrophin dosing days	N	N	N
MTHFR (Y/N)	N	N	Y
Catheter used	N	N	N
HCG name	N	N	N
Eggs collected	N	N	N
Mature eggs inseminated	N	Y	N
Mature eggs inseminated out of eggs collected ratio	N	N	N
Transfer grade/morphology	Y	Y	N
Patient type (oocyte recipient/standard)	Y	Y	Y
Donor age (years)	N	N	N
Gonadotrophin total dose (IU)	N	Y	N
Oocyte recipient/patient age interaction	N	N	N
Embryo rank/patient age interaction	N	N	N
Embryo rank/oocyte recipient interaction	N	N	N
Embryo rank/donor age interaction (where applicable)	N	N	N
Embryo rank/day of embryo transfer interaction	N	N	N
Embryo rank/patient age/oocyte recipient interaction	N	N	N
Embryo rank/transfer grade interaction	N	Y	N
Embryo rank/transfer stage interaction	N	N	N
Embryo rank/day of embryo transfer and transfer grade interaction	N	N	N
Embryo rank/day of embryo transfer and transfer stage interaction	N	N	N

AFC = antral follicle count; AMH = anti-Müllerian hormone; BMI = body mass index; HCG = human chorionic gonadotrophin.

OOCYTE RETRIEVAL, DENUDATION AND INTRA-CYTOPLASMIC SPERM INJECTION

Female sedation was achieved with a combination of propofol (Braun, Germany), fentanyl (Auden McKenzie, UK) and midazolam (Hamelyn, UK), and transvaginal ultrasound-guided oocyte retrieval took place approximately 36 h post human chorionic gonadotrophin injection (10,000 IU; Pregnyl; Organon, UK; or Ovitrelle; Merck Serono) or agonist trigger (Buserelin 0.5 ml subcutaneous; Suprefact, Sanofi SA, France), using an aspiration needle (Vitrolife, Sweden) connected to a vacuum pump (Rocket Medical, UK). Oocyte–cumulus complexes were recovered from follicular aspirates using a stereomicroscope in a class II hood with a heated stage, washed and cultured in Ferticult IVF medium (Fertipro, Belgium) at 5% CO_2 in air, 37.0°C, and maximum humidity, in standard small volume box or flatbed incubators (Galaxy 48R, New Brunswick, UK; Miri, ESCO, Japan).

Oocytes allocated for intracytoplasmic sperm injection (ICSI) were cultured for 2–4 h before cumulus cell denudation with 15–20 IU/ml cumulase (Origio, Denmark) in the same medium and complete removal of the coronae radiatae with a 140 µm pipette (EZ Squeeze; Research Instruments, UK). Oocytes at the metaphase II stage underwent insemination by ICSI within 2 h of denudation, following which they were placed in the EmbryoScope. Oocytes allocated for IVF were inseminated following sperm preparation using SupraSperm density gradient (Origio, Denmark) and washing in Ferticult IVF medium (Fertipro) at a concentration of 0.2 mmol/l, between 3 and 6 h post oocyte recovery. Culture was performed in standard incubators for 18 ± 1 h before fertilization was assessed. The sperm preparation method was the same for IVF and ICSI.

EMBRYO CULTURE AND INCUBATION

For TLI, following ICSI or IVF, oocytes or zygotes, respectively, were placed individually in microwells of EmbryoSlides (Vitrolife, Sweden) in 25 µl Global IVF medium (LifeGlobal) supplemented with 10% dextran serum supplement (Irvine Scientific); the wells were overlaid with 1.4 ml mineral oil (Fertipro, Belgium) and the slides were placed in the EmbryoScope at 37.0°C in 5.5% CO_2, 5% O_2 and 89.5% N_2 for up to 6 days. EmbryoSlides were prepared with medium and oil that had equilibrated overnight. The built-in microscope was used to acquire images of each fertilized oocyte every 10–20 min through seven focal planes.

Selection of the embryos was based on time-lapse algorithm ranking (A to D) and morphological assessment was recorded for all transferred blastocysts using a scoring system modified from the *Gardner and Schoolcraft (1999)* annotation of trophectoderm and inner cell mass morphology where scores AA/AB/BA, etc. were replaced by numbers: 1:1/1:2/2:1, etc., as per the Istanbul consensus recommendation (*The Istanbul Consensus, 2011*).

EVALUATION OF TIME-LAPSE IMAGES

Time-lapse images were collected for the duration of the culture period, until embryo transfer. The images were used for the assessment of fertilization following ICSI and *in vitro* embryo development. For ICSI, the time of insemination was programmed into the EmbryoScope as the time-point midway through the ICSI procedure. For IVF, the time of insemination was recorded as the time spermatozoa were added to the oocytes. Because of the difference in timing of sperm penetration in ICSI versus IVF, the designation of time post insemination (hpi) was based on pronuclei fading (PNf) after carefully controlled annotation. For ICSI, the mean time was 23.23 ± 3.7 h (n = 2547 zygotes); for IVF, the mean was 25.18 ± 3.6 h (n = 785). Hence ICSI zygote PNf occurred on average 1.95 h before IVF embryos. This difference in the modelling is accounted for as described below.

The EmbryoViewer image analysis software (Vitrolife) was used to log and display the precise timing of developmental events as they were annotated by the embryologists studying the time-lapse images. The morphokinetic variables of interest have been described in detail previously (*Campbell et al., 2013a, 2013b*; *Fishel et al. 2017*). All times were recorded in hpi. Following implementation of an in-house-derived time-lapse algorithm to rank embryos according to likelihood of live birth, the algorithm was used prospectively for selection of blastocysts for transfer. For this study, blastocyst ranking depended upon annotating for the relative time to the start of blastulation (rtSB) and the duration of blastulation (dB), with the following weighting: rank A = rtSB ≤ 93.1 h; B: rtSB > 93.1 h, dB ≤ 12.5 h; C: rtSB > 93.1 h, dB > 12.5 h. D was scored for those embryos in which the start of blastulation could not be annotated; this may occur due to obscuring fragmentation, presence of multiple fluid-filled vacuoles, or anomalous kinetics. As the algorithm's morphokinetic variable, tSB, is based on time from insemination, and as we observed a 1.95 h delay for IVF embryo development (unpublished data) compared with ICSI, the algorithm was adjusted for IVF embryos as follows in order to generate the ranking (A–D): RelSBIVF = tSB – 1.95 h.

EMBRYOLOGY ANNOTATION PROTOCOLS AND QUALITY CONTROL

Following training in annotation and competency assessment, CARE embryologists participate in regular quality assurance (QA) exercises and use a centralized annotation QA protocol whereby example embryos are annotated by each practitioner and their values are compared with those of their colleagues. Intra correlation coefficients (ICC) are calculated for each morphokinetic value. Annotation quality is considered assured where the ICC is greater than 0.9, demonstrating close correlation between practitioners, and competency in annotation.

EMBRYO SELECTION AND TRANSFER

Following TLI, embryos were objectively selected using user-defined time-lapse algorithms programmed into the 'Compare and Select' software as described above. All embryos were annotated before decision on transfer. Rank A was given the highest priority while rank D had the lowest. Morphology was scored at the time of transfer, but for decision on transfer, morphology was considered only secondary to the morphokinetic algorithm.

Embryo transfer was performed using a Wallace (UK) embryo transfer catheter under ultrasound guidance.

STATISTICS

The primary outcome measurement was live birth, i.e. the delivery of one or more babies; and the secondary outcome was the incidence of miscarriage (including HCG-determined implantation which did not result in a clinical pregnancy and clinical loss, defined as a loss of pregnancy following fetal heart detection on ultrasound). Separate multi-variable logistic regression models were fitted to the study data in order to assess the effects of embryo rank on each outcome of interest. A logistic regression analysis models the probability of the binary outcome as a function of the supplied explanatory variables. To explicitly control for differences in the patient populations between the embryo rank groups, potential confounding variables from the available clinical data were also considered as other explanatory variables in the models.

To choose which of the available variables to include as covariates in each model, a forward–backward stepwise variable selection procedure was performed. This procedure started with an initial model that included just an intercept term and new explanatory variables were added (and removed) one at a time to look for the model that has the best value of the Akaike information criterion (AIC) – a measure of model fit that has a penalty for the number of parameters in the model. TABLE 1 shows the variables made available to the variable selection procedure and those that were selected to be in the final model for each outcome.

A number of interactions ('effect modifications') were made available to the variable selection procedure. An interaction allows the level of one or more variables to change the effect of another variable. Any combination of variables may interact with each other, resulting in an enormous number of potential interactions. To make the variable selection and model fitting practicable, the interactions were limited to the following:

- Patient age with an indicator for whether the patient was an oocyte recipient, to allow for the possibility that the patient's age is less (or even more) important when they have received an egg donation.
- Embryo rank with each of patient age, oocyte recipient indicator, donor age (where applicable), day of embryo transfer, transfer grade and transfer stage (number of cells) and day of embryo transfer and morphology. These interactions were included to allow for the possibility that the sizes and directions of the embryo rank effects vary for different groups of patients.
- A three-way interaction between embryo rank, oocyte recipient status and patient age was also made available. This was included because the corresponding two-way interaction without embryo rank was being included. Importantly, these interactions were included to allow for the possibility that the size and direction of the embryo rank effects vary for different groups of patients.

Fitted final models were used to provide estimates of the directions and sizes of the effects of interest (presented as odds ratios [OR] which describe the relative difference in the odds of a live birth or clinical miscarriage between different treatment cycles) for each outcome (TABLE 2). The estimated effect sizes are accompanied by profile likelihood confidence intervals (CI), which quantify the uncertainty in the estimates arising from the sample data. Likelihood ratio tests (LRT) are provided to assess the overall significance of each variable, across the multiple groups of a categorical variable. A LRT tests the null hypothesis that including the variable in question does not improve the model fit (as measured by the likelihood).

All analyses were performed using the statistical software package R version 3.3.1 (*R Core Team, 2016*). The logist package was used to implement Firth's penalized maximum likelihood logistic regression method (*Heinze and Ploner, 2016*) when analysing clinical miscarriage.

RESULTS

A total of 843 single blastocyst transfers were examined for LB according to their TLI algorithm value of the transferred embryos. The mean ages of patients were 34.8 (n = 373), 35.4 (n = 297), 34.8 (n = 93) and 37.1 (n = 80) for embryos with algorithm ranks of A, B, C and D, respectively. An assessment of the value of a hierarchical ranking of embryos using all the available clinical data was undertaken, as shown in TABLE 1. A full data set was available for 781 transfers, included in a detailed analysis; summary statistics for each continuous or discrete variable were considered in the analysis versus embryo rank and are shown in TABLE 2. Of the 781 transfers, 354 (45.3%) had an embryo ranked A, 273 (35.0%) ranked B, 86 (11.0%) ranked C, and 68 (8.7%) ranked D. There were 329 (42.1%) live births; 186 (52.5%) from transfers with an embryo ranked A, 107 (39.2%) from rank B, 27 (31.4%) from rank C, and 9 (13.2%) from D-ranked embryos. There was strong evidence of an effect of embryo rank on the odds of live birth. Embryos of rank D were less likely to result in a live birth than embryos of rank A (OR 0.3046; 95% CI 0.129, 0.660; $P < 0.010$), and similarly embryos of rank D were less likely to result in a live birth than embryos of rank B (OR 0.428; 95% CI 0.190, 0.963; $P < 0.01$). Embryos ranked B were less likely to result in a live birth than those ranked A (OR 0.7114; 95% CI 0.505, 1.001; $P = 0.0101$), and embryos ranked C were less likely to result in a live birth than those ranked A (OR 0.6501, 95% CI 0.373, 1.118; $P = 0.0101$). Overall, the data provide strong evidence of an effect of embryo rank on the odds of live births (LRT p-value = 0.0101). There was no evidence of an independent effect of embryo rank on the odds of clinical miscarriage.

The role of morphology assessment was also examined; in the absence of TLI data, this would have been the primary factor in deciding which embryo to transfer. The highest incidence of live birth was achieved with blastocysts graded '2:2' when compared with other morphology grades (FIGURE 1). The comparison showed that selecting for an embryo grade 2:2 for transfer is associated with an increase in the odds of a live birth compared with grades 1:1/1:2/2:1 (OR 0.6795; 95% CI 0.485, 0.988), or with grades 2:3/3:2/3:3; OR 0.3181; 95% CI: 0.171, 0.573).

DISCUSSION

In summary, using the EmbryoScope for culture to the blastocyst stage, coupled with objective embryo selection criteria based on a morphokinetic algorithm, embryos can be successfully ranked based on their chance of achieving a live birth. An embryo rank of D is estimated be associated with a 69.5%

TABLE 2 SUMMARY STATISTICS FOR EACH CONTINUOUS OR DISCRETE VARIABLE CONSIDERED IN THE ANALYSIS VERSUS EMBRYO RANK. THE MEAN (AND SD = STANDARD DEVIATION) AND MEDIAN (AND RANGE), BY RANK

Variable	Summary	A (*n* = 354)	B (*n* = 273)	C (*n* = 86)	D (*n* = 68)	Total (*n* = 781)
Patient age (years)	Mean (SD)	35.4 (5.11)	36.0 (4.96)	36.2 (5.13)	36.5 (5.20)	35.8 (5.07)
Patient age (years)	Median (range)	35.0 (22–48)	35.0 (24–49)	36.0 (25–50)	36.5 (26–47)	35.0 (22–50)
Total previous cycles	Mean (SD)	3.2 (2.02)	3.1 (1.62)	3.6 (2.52)	3.9 (2.42)	3.3 (2.00)
Total previous cycles	Median (range)	2.0 (2–16)	2.0 (2–12)	3.0 (2–18)	3.0 (2–16)	2.0 (2–18)
Total previous miscarriages	Mean (SD)	0.3 (0.61)	0.3 (0.69)	0.4 (0.84)	0.4 (0.77)	0.3 (0.68)
Total previous miscarriages	Median (range)	0.0 (0–4)	0.0 (0–5)	0.0 (0–5)	0.0 (0–3)	0.0 (0–5)
Maximum endometrial thickness (mm)	Mean (SD)	10.9 (2.60)	10.6 (2.28)	10.9 (2.62)	10.5 (2.16)	10.8 (2.46)
Maximum endometrial thickness (mm)	Median (range)	10.6 (6–20)	10.2 (6–19)	10.2 (6–17.5)	10.5 (7–19.5)	10.4 (6–20)
Donor age (years)	Mean (SD)	29.3 (3.98)	29.6 (3.78)	30.8 (3.75)	30.3 (4.38)	29.6 (3.92)
Donor age (years)	Median (range)	30.0 (20–36)	30.0 (21–35)	31.0 (22–37)	29.0 (24–38)	30.0 (20–38)
Duration of infertility (years)	Mean (SD)	3.3 (2.47)	3.0 (2.27)	2.9 (2.38)	3.6 (3.10)	3.2 (2.46)
Duration of infertility (years)	Median (range)	3.0 (0–12)	3.0 (0–16)	2.0 (0–15)	3.0 (0–21)	3.0 (0–21)
Gonadotrophin total dose (IU)	Mean (SD)	2283.1 (981.18)	2294.3 (1112.53)	2439.2 (1075.45)	2862.5 (1199.18)	2356.2 (1069.58)
Gonadotrophin total dose (IU)	Median (range)	2250.0 (450–5400)	2250.0 (450–6000)	1800.0 (675–5400)	2700.0 (525–5400)	2250.0 (450–6000)
Gonadotrophin dosing days	Mean (SD)	10.2 (2.42)	9.8 (2.73)	10.3 (2.31)	10.6 (1.72)	10.1 (2.48)
Gonadotrophin dosing days	Median (range)	12.0 (3–12)	10.0 (3–12)	12.0 (3–12)	10.5 (6–12)	10.0 (3–12)
Eggs collected	Mean (SD)	12.2 (5.72)	11.2 (5.52)	9.8 (5.45)	7.2 (3.71)	11.1 (5.65)
Eggs collected	Median (range)	11.0 (1–34)	11.0 (1–30)	9.0 (2–26)	7.0 (1–21)	10.0 (1–34)
Mature eggs inseminated	Mean (SD)	9.8 (4.71)	9.2 (5.00)	8.1 (4.68)	5.3 (3.56)	9.0 (4.88)
Mature eggs inseminated	Median (range)	9.0 (0–28)	9.0 (0–28)	7.0 (1–25)	4.5 (1–18)	8.0 (0–28)
Mature eggs inseminated out of eggs collected ratio	Mean (SD)	0.8 (0.17)	0.8 (0.19)	0.8 (0.18)	0.7 (0.24)	0.8 (0.19)
Mature eggs inseminated out of eggs collected ratio	Median (range)	0.9 (0–1)	0.9 (0–1)	0.9 (0.25–1)	0.8 (0.125–1)	0.9 (0–1)
Oocyte recipient patient age (years)	Mean (SD)	41.4 (4.01)	41.4 (4.74)	41.6 (5.61)	40.0 (5.41)	41.3 (4.55)
Oocyte recipient patient age (years)	Median (range)	42.5 (24–48)	42.0 (27–49)	44.0 (29–50)	40.5 (26–47)	42.0 (24–50)
Non-oocyte recipient patient age (years)	Mean (SD)	33.6 (3.88)	34.4 (3.72)	34.6 (3.78)	35.6 (4.79)	34.2 (3.94)
Non-oocyte recipient patient age (years)	Median (range)	34.0 (22–43)	34.0 (24–46)	35.0 (25–41)	35.5 (26–45)	34.0 (22–46)

decrease in the odds of a live birth compared with an embryo of rank A. The 95% CI for this comparison ranges from an 87.1% decrease to a 34.0% decrease in the odds. The analysis also highlighted the weakness of dependence on conventional morphology alone as a selection tool. For example, a grade 2:2 blastocyst, which is the equivalent of the *Gardner and Schoolcraft (1999)* ranking BB, results in live birth at a significantly higher frequency than embryos graded 1:1/1:2 or 2:1, which contradicts previous publications (*Gardner and Schoolcraft, 1999; Gardner et al., 2000;* Istanbul Consensus, 2011). While TLI algorithms are objective and likely to achieve greater consistency in embryo selection, static morphology assessments remain subjective and prone to variability. Additionally, morphology grading is likely to reflect a limited number of single daily time-points when the grade is assigned, whereas TLI can provide photographs of the embryo at frequent intervals throughout culture; this in turn provides the opportunity to witness and evaluate changes in embryo morphology over time and therefore avoid the unreliability of a single-moment, subjective morphology grading. One of the limitations of most TLI studies is the comparison between TLI and conventional incubators for culturing embryos, irrespective of the use of algorithms; this type of comparison leaves open the question of whether uninterrupted incubation alone is an advantage, whether or not morphokinetics are considered for embryo selection. This study compares embryos cultured only in the EmbryoScope from Day 0 (ICSI) or Day 1 (IVF) up to Day 5 of development.

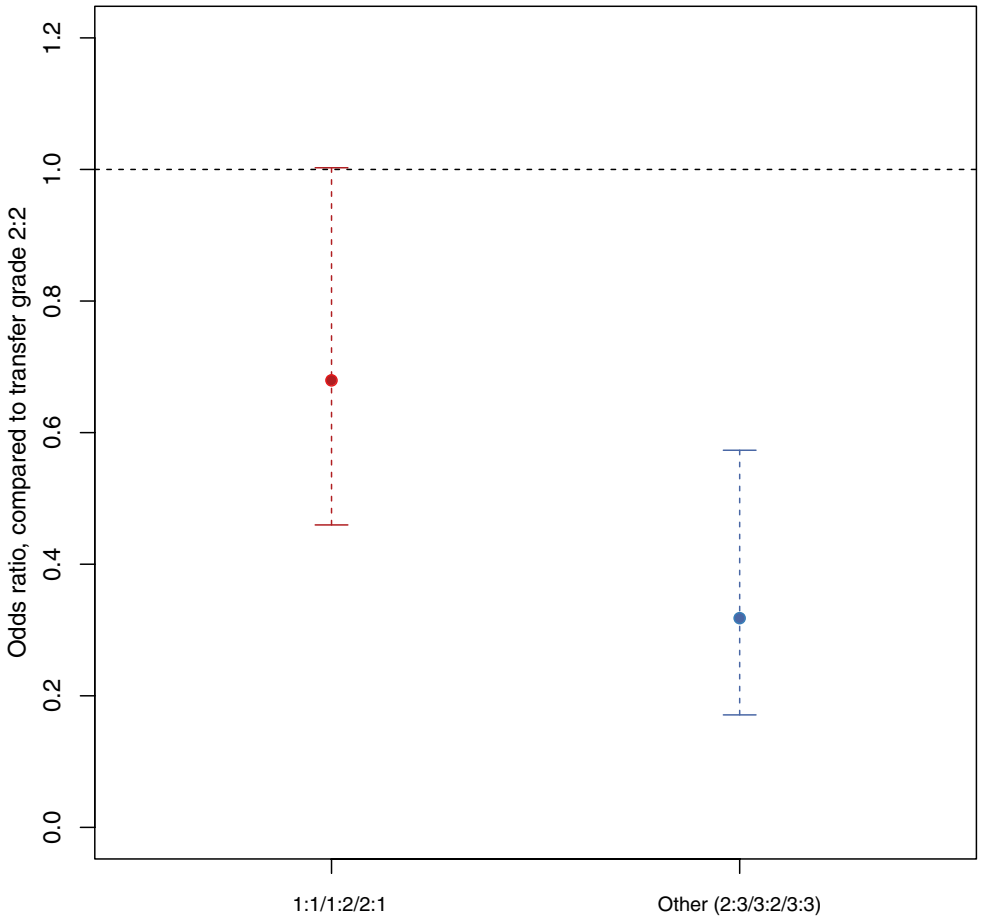

FIGURE 1 Model estimated odds ratios, comparing the occurrence of live birth between transfer grades. The bars indicate 95% confidence intervals.

It also includes single blastocyst transfers only, to avoid any ambiguity of the effects of more than one embryo transferred. The results demonstrate the value of an objective algorithm for embryo selection. The main limitations of this study are, first, that the analysis was undertaken to validate the effectiveness of an in-house-derived algorithm and this algorithm may not produce similar results in other settings (*Freour et al., 2015; Liu et al., 2016*). Secondly, the efficacy of this ranking system remains to be demonstrated prospectively. Further, it is not possible to account for any clinical or physiological differences not contained within the available dataset and therefore this investigation may not have eliminated all confounding effects in this non-randomized study.

TLI has been used in clinical practice for over 10 years (*Hlinka et al., 2012; Lemmen et al., 2008*) with several recent reviews (*Armstrong et al., 2015*; *Chen et al., 2017; Kaser & Racowsky, 2014; Polanski et al., 2014; Racowsky et al., 2015*). Systematic reviews have either considered studies comparing TLI incubation to conventional incubation, or the overall impact of TLI as compared with conventional methods, or the potential effect of using morphokinetics for embryo selection. Van Loendersloot *et al.* (2014) applied a retrospective multivariable clinical pregnancy prediction model to rank embryos following Day 3 transfer, distinguishing those embryos with high, moderate or low implantation potential. Petersen *et al.* (2016) also used a morphokinetic algorithm for embryos transferred on Day 3 to predict blastulation based on data from 24 clinics over a 5-year period. However, apart from patient age, and whether IVF or ICSI was performed, embryos were treated as independent variables; there is concern regarding the validity of such studies because cohorts of embryos should not be considered independent. When associating embryological data with clinical outcome, all potential clinical confounders must be considered (*Fishel et al., 2017; Kirkegaard et al., 2016*).

Several studies have, however, reported on the use of TLI as a potential prognosticator in clinical practice (*Adamson et al., 2016; Chen et al., 2016; Kong et al., 2016; Liu et al., 2016; Milewski et al., 2015; Mizobe et al., 2016; Rubio et al., 2014; VerMilyea et al., 2014; Wu et al., 2016; Yang et al., 2014*). Other studies have disagreed (*Freour et al., 2015; Wu et al., 2016*). Given the complex nature of the effects of culture conditions and the milieu on preimplantation development, in addition to their inherent genetic and chromosomal complements, it is difficult to pinpoint any single feature that may directly impact outcome, especially because development of the implanting embryo to a live baby depends also on the maternal environment. A few studies have purported to relate discriminating morphokinetics of euploid and aneuploid embryos (*Campbell et al., 2013a, 2013b; Minasi et al., 2016; Vera-Rodriguez et al., 2015*) although

this has been disputed (*Rienzi et al., 2015*). However, *Bronet et al. (2015)* even found distinctive morphokinetic differences between male and female embryos. More recently *Mumusoglu et al. (2017)*, studying morphokinetics and the prediction of ploidy status when patient and ovarian stimulation–related factors were taken into account, concluded that aneuploid embryo development appears to be delayed at post-cleavage stages, but that the predictive ability was 'low to moderate'. Of previously reported cut-off points for various TLM parameters, they only noted tSB within 96.6 h of insemination as having significant predictive ability (*Campbell et al., 2014*). *Kong et al. (2016)* reported a relationship between early cell division behaviour and developmental potential with elongation or shortening of the cell cycle affecting cell number. This study concluded that by excluding such embryos, the incidence of implantation and live birth following Day 3 transfer of embryos increased when cell number was maximal. Recently *Ottolini et al. (2017)*, in an important study using TLI and genome-wide SNP genotyping and meiomapping of both polar bodies, analysed tripolar and other abnormal mitoses demonstrating that failure to coordinate the cell cycle in early cleavage and regulation of centrosome duplication is a major cause of human preimplantation developmental arrest *in vitro*.

Morphological evaluation of the embryos at specific time-points has been the method of choice for embryo selection for decades (*Cummins et al., 1986; Fishel et al., 1983, 1983*), although its limitations have later been recognized (*Fehilly et al., 1985; Guerif et al., 2007; Hartshorne et al., 1991; Racowsky, 2009*). Blastocysts also undergo a normal cycle of collapse and re-expansion and are often difficult to grade reliably. Although they can be reassessed at other time-points (The Istanbul Consensus, 2011), TLI highlights the transient nature of certain morphologies and thus a source of weakness of morphology-based grading as a prognosticator. A number of studies have found weak correlation between blastocyst morphology and chromosomal abnormalities, including those incompatible with post-implantation development (*Fragouli et al., 2010; Schoolcraft et al., 2010*).

In the most recent meta-analysis on five randomized controlled trials using TLI algorithms, *Pribenszky et al. (2017)* reported a significantly higher incidence of ongoing clinical pregnancy and live birth, and a significantly lower incidence of early pregnancy loss following time-lapse incubation and algorithm-based embryo selection compared with conventional culture with embryo selection based on single time-point morphology. The studies included a heterogeneous patient population, days of transfer, the way the visual information from the time-lapse devices was used to support embryo evaluation and end-points, and as such the quality of the evidence was deemed moderate to low owing to inconsistencies across the studies. Indeed, the studies in general did not include a comprehensive clinical confounder analysis. *Motato et al. (2016)* used the timing of expanded blastocyst formation (tEB) as the primary variable at ≤122.9 h and the synchronicity of the third round of cleavage divisions (s3 = t8 – t5) as the secondary variable setting the optimal range of ≤5.67 h. *Motato et al. (2016)* used these data to divide embryos into four categories (A–D), with a decreasing implantation potential (from 72.2% for A to 39.7% for D). However, there are now many different algorithms of apparent efficacy, but in some laboratories advantages have not been proven, as comprehensively reviewed recently by *Milewski and Ajduk (2017)*.

In conclusion, this study demonstrates the unique live birth capacity of individual blastocysts based on an algorithm that incorporates time to start and duration of blastulation. These parameters must be carefully annotated by embryologists, with tight quality control on annotation principles, and within a single culture system. This study further demonstrates the advantage of objective data, which can be revisited any time (as TLI images, for example) without embryo disruption, over the capricious and subjective morphological scoring at one or more discrete time-points. The study also highlights the need to ensure that analyses do not treat embryos as independent variables because this can lead to erroneous conclusions; it also argues in favour of inclusion of full clinical data in analyses of this type before drawing conclusions on clinical impact. The data importantly implicate a relatively simple algorithm to rank and therefore select embryos in clinical practice to improve the chance of a live birth. Although more high-quality evidence, such as large, well-controlled prospective randomized studies, are needed to definitively demonstrate the value of TLI, further research into other cellular processes such as chromosome segregation, cytoskeleton function and energy metabolism are required to reduce embryo wastage and unnecessary embryo transfers and cryopreservation and enable selection of only those embryos with the capacity to reach full term.

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6.0 Specific aim 5.

To establish best laboratory practice for time lapse imaging, a) by engaging with the scientific community to develop and propose guidelines on the nomenclature and annotation of dynamic human embryo monitoring; and b) developing a resource for embryologists using time lapse imaging and c) to help develop international IVF laboratory performance indicators and IVF culture conditions in general.

Encapsulated in this specific aim are four book chapters, the study proposing standard nomenclature and practice for time lapse imaging in human IVF, comprehensive standard operating procedures for a group of 11 UK IVF laboratories (CARE Fertility), a published Atlas of Time Lapse Embryology and two consensus documents developing performance indicators and guidelines on IVF culture conditions.

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***Alison Campbell** and Simon Fishel. Altas of Time Lapse Embryology. CRC Press, 2015.* This book received a 'Highly Commended' award in the BMA Medical Book Awards 2016.

ESHRE Special interest Group of Embryology, Alpha Scientists in Reproductive medicine. The Vienna Consensus: Report of an expert meeting on the development of ART laboratory performance indicators.

There is only one thing that is truly important in an IVF lab: Everything. Cairo Consensus guidelines on IVF culture conditions. 2019 in press

Here, my personal contributions were;

- To author three book chapters pertaining to time lapse and morphokinetic embryo selection, and to co author another.
- To assist in scoping the proposed guidelines project, to write a proportion of the text, to edit the manuscript and co-first author this well cited manuscript, with international colleagues.

- To work with the publisher and co editor to scope the Atlas, the first of its kind, as a resource for scientists and clinicians working within the field of IVF.
- To develop a strict standard operating procedure, and quality assurance process to provide robust data and clinical results. (See appendix 3)
- To collaborate with international IVF professionals and professional bodies to develop IVF laboratory performance indicators.
- To collaborate with international IVF professionals to develop guidelines for ART laboratory culture conditions.

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ESHRE PAGES

The Vienna consensus: report of an expert meeting on the development of art laboratory performance indicators[†,‡]

ESHRE Special Interest Group of Embryology[1,*] and Alpha Scientists in Reproductive Medicine[2,*]

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STUDY QUESTION: What are appropriate performance indicators (PIs) for ART laboratories for use in monitoring 'fresh' IVF and ICSI cycles?

SUMMARY ANSWER: Minimum performance (competence) levels and aspirational (benchmark) values were recommended for a total of 19 indicators, including 12 key PIs (KPIs), five PIs and two reference indicators (RIs).

WHAT IS ALREADY KNOWN: PIs are necessary for systematic monitoring of the laboratory and an important element within the Quality Management System. However, there are no established PIs for ART laboratories and there is very little evidence on the topic.

STUDY DESIGN, SIZE, DURATION: This is the report of a 2-day consensus meeting of expert professionals. As a starting point for the discussion, two surveys were organized to collect information on indicators used in IVF laboratories. During the meeting, the results of the surveys, scientific evidence (where available), and personal clinical experience where integrated into presentations by experts on specific topics. After presentation, each proposed indicator was discussed until consensus was reached within the panel.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Expert professionals representing different countries and settings convened in the consensus meeting.

MAIN RESULTS AND THE ROLE OF CHANCE: The paper is divided in two parts: the workshop report and the recommendations of the expert panel. The second part reflects the discussion on each of the indicators, with the agreed definition, competence level and benchmark value for each of the 19 indicators, including 12 KPIs, 5 PIs and 2 RIs.

LIMITATIONS, REASONS FOR CAUTION: The KPIs are mainly based on expert opinion. Future research may warrant an update of the recommended KPIs, their definition and the competence level and benchmark values.

WIDER IMPLICATIONS OF THE FINDINGS: Based on the information presented, each ART laboratory should select its own set of KPIs founded on laboratory organization, and processes.

STUDY FUNDING/COMPETING INTEREST(S): The consensus meeting and writing of the paper was supported by funds from ESHRE and Alpha. Alpha gratefully acknowledges the following organizations for their financial support, through the provision of unrestricted educational grants: Global Fertility Alliance, Merck, Origio and Vitrolife. There are no conflicts of interest to disclose,

Key words: embryo / IVF / KPI / oocyte / performance indicator / sperm

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[‡]This article has been co-published with permission in *HROpen* and *Reproductive Biomedicine Online*.

WHAT DOES THIS MEAN FOR PATIENTS?

Performance indicators (PI) are seen as a good way to check that healthcare is working well and within safety guidelines. There are not any existing PIs set across the board for IVF laboratories. This report looks at PIs for laboratories, and is based on a 2 day professional meeting and two surveys which aimed to find out more about how IVF laboratories are currently working. The aim was to set up key PI (KPIs) for IVF laboratories and an expert panel made some recommendations.

The report looked at a number of processes in an IVF laboratory including egg collection, how eggs are evaluated and sperm is assessed, fertilization rates after IVF and ICSI (where the sperm is injected directly into the egg), how well embryos develop, freezing rates and treatment outcomes. For each of these processes KPIs were defined.

The expert panel concluded that it was important for each laboratory to monitor and check their own performance regularly. The panel outlined good practice and suggested a list of 19 PIs for IVF laboratories. The panel also suggested that in the future staff at each laboratory should select their own indicators based on the way that they worked.

Introduction

Performance indicators (PIs) are objective measures for evaluating critical healthcare domains (patient safety, effectiveness, equity, patient-centeredness, timeliness and efficiency) (Kohn *et al.*, 2000). In the setting of a clinical laboratory, quality indicators are necessary for systematically monitoring and evaluating the laboratory's contribution to patient care (ISO15189-2012) and they represent an important element within the Quality Management System (QMS) (Mortimer and Mortimer, 2015; ESHRE Guideline Group on Good Practice in IVF Labs *et al.*, 2016). Currently, there are no established PIs for ART laboratories, and there is very little published evidence on the topic.

Any PI should be reliable and robust, and routine data collection for the indicator should be straightforward. Furthermore, the biological or technical process to be monitored should be defined, and relevant qualifiers, confounders and endpoints should be identified. Key PIs (KPIs) are indicators deemed essential for evaluating the introduction of a technique or process; establishing minimum standards for proficiency; monitoring ongoing performance within a QMS (for internal quality control (IQC), external quality assurance (EQA)); benchmarking and quality improvement. In general, the results of a series of KPIs will provide an adequate overview of the most important steps in the IVF laboratory process (Salinas *et al.*, 2010).

The aim of the consensus meeting and report was to establish KPIs for ART laboratories for the use in monitoring 'fresh' IVF and ICSI cycles and provide the basis for several of the quantitative performance criteria needed to create competency profiles for Clinical Embryologists. More specifically, the purpose was to achieve an international consensus regarding: first, a minimum list of IVF laboratory indicators and KPIs that in the future can be further extended and/or revised; second, specific definitions for these indicators (including necessary case inclusion/exclusion criteria; and calculation formulae); and third, recommended values for each KPI (minimum 'competency' limit; and 'aspirational goal' benchmark).

Based on the information presented here, each laboratory should develop its own set of KPIs founded on laboratory organization and processes, and develop a systematic, transparent and consistent approach to data collection and analysis and calculation of KPIs (Mayer *et al.*, 2003; Salinas *et al.*, 2010; Mortimer and Mortimer, 2015; ESHRE Guideline Group on Good Practice in IVF Labs *et al.*, 2016).

Metholdology

This report is the result of a 2-day consensus meeting of expert professionals (participants are listed in Table I). As a starting point for the discussion at the meeting, two surveys were organized to collect information on indicators used in IVF laboratories. The first, the 'Alpha survey', was sent to national and international societies of ART laboratory directors and

Table I Consensus workshop participants and contributors.

Name	Affiliations
Susanna Apter	Fertilitetscentrum Stockholm, Sweden
Basak Balaban	American Hospital of Istanbul, Turkey
Alison Campbell*	CARE Fertility Group, UK
Jim Catt	Optimal IVF, Melbourne, Australia
Giovanni Coticchio	Biogenesi, Monza, Italy
Maria José de los Santos*	IVI Valencia, Valencia, Spain
Sophie Debrock*	Leuven University Fertility Centre, Leuven, Belgium
Thomas Ebner*	Kepler University, Linz, Austria
Stephen Harbottle	Cambridge IVF, UK
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Sharon Mortimer	Oozoa Biomedical, Vancouver, Canada
Zsolt Peter Nagy	Reproductive Biology Associates, Atlanta, USA
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Nathalie Vermeulen	ESHRE Central Office, Grimbergen, Belgium

*Presenters at the consensus workshop.

Clinical Embryologists, and to the members of the ESHRE committee of national representatives. Eighteen responses (with opinions from Australia, Austria, Belgium, Bulgaria, Canada, Croatia, France, Germany, Ireland, Italy, Japan, Slovenia, Sweden, South Africa, Turkey, UK and USA) out of 34 sent were received, and its results informed the expert panel on minimum expected, or competence, values (i.e. values that any laboratory should be able to achieve), and aspirational, or benchmark, values (i.e. values that can be employed as a best practice goal), for a range of quality indicators. Where possible, responses were based on standardized information (national collected data, or large data sets), but in most countries such data are not available. Another survey, the 'ESHRE survey', provided information on current practice (how many KPIs are measured, frequency of measurement, characteristics of a reference population for KPIs) and the degree of importance of some indicators. This survey was sent to 2413 members of the ESHRE Special Interest Group (SIG) of Embryology, and 384 responses were received. In addition, where relevant, published data were collected from a literature search and summarized, although for most indicators, especially in ART, there is a general lack of evidence to support their importance, scientific soundness and usefulness (Shahangian and Snyder, 2009).

During the consensus meeting, the results of the surveys, scientific evidence and personal clinical experience were integrated into presentations by experts on specific topics. For each indicator, information was presented in a fixed format: definition, rationale, qualifiers, formula, data sources, KPI strengths and weaknesses, frequency of data collection and reference values for minimum expected and target value based on 50 and 75 percentile values, respectively. After the presentation for the topic, each proposed indicator was discussed until consensus was reached within the group.

After the meeting, a report was prepared describing the presentations (workshop report) and the consensus points. After approval of the report by the meeting participants, the national and international societies that contributed to the questionnaires were invited to review the report and submit comments. The final version of the manuscript was approved by the Executive Committees of ESHRE and Alpha before publication.

This paper is divided into two parts: the workshop report and the recommendations of the expert panel.

Results (Workshop report)

Effects of ovarian stimulation on embryology parameters

The methods of ovarian stimulation have been evolving since the earliest days of clinical IVF, in the search for the best stimulation protocol. With that goal in mind, there has been an enormous effort to develop the best pharmaceuticals and protocols, but in practice, economic factors as well as prevailing opinion can influence treatment decisions beyond consideration of the patient's endocrine background.

Despite the thousands of smaller studies on this subject in the literature, there are very few large multicentre RCTs beyond those organized by pharmaceutical companies, and these do not consider 'non-standard' groups of patients. Furthermore, the meta-analyses that have been conducted are often unclear about their inclusion criteria. As a result, the prevailing approach is to use what works within each clinic, and is often uncorrected for the patients' weight, BMI or endocrine background. Some clinics also do not monitor cycles, for financial reasons, which can have an influence on the cycle outcome.

In general, a 'good' stimulation is one that produces a homogeneous cohort of mature oocytes, with the least inconvenience and risk to the patient, and results in the birth of a healthy singleton. From the Clinical Embryologist's perspective, a good stimulation results in the retrieval of well-expanded cumulus-oocyte complexes (COCs), as is expected from each follicle >14 mm in diameter, with a high proportion of metaphase II (MII) oocytes (Scott *et al.*, 1989; Ectors *et al.*, 1997; Nogueira *et al.*, 2006). On the other hand, a poor stimulation, caused for example by sub-optimal decisions regarding timing or stimulation dose, is one that may result in a high rate of abnormal COC morphology observed at the time of oocyte retrieval, possibly resulting in an increased rate of abnormal fertilization (e.g. one pronucleus [PN], 3PN, etc.) and/or decreased rate of normal fertilization and an increased aneuploidy rate (Soares *et al.*, 2003).

Aggressive ovarian stimulation has effects on the patient's well-being, by increasing the risk of ovarian hyperstimulation syndrome, (Delvigne, 2009; Rizk, 2009) as well as on the endometrium and the ovaries. There are studies showing an increased likelihood of embryo aneuploidy in connection with aggressive ovarian stimulation, even in patients younger than 35 years, including post zygotic segregation errors and maternal segregation errors (Baart *et al.*, 2007; Haaf *et al.*, 2009), as well as having a negative impact on the maintenance of genomic imprints during early embryogenesis (Fauque *et al.*, 2007; Denomme and Mann, 2012; Saenz-de-Juano *et al.*, 2016). It has been shown that in patients belonging to either high or low strata of antimüllerian hormone levels an inverse correlation exists between the daily dose of recombinant human FSH used in the stimulation and the proportion of blastocyst formation (Arce *et al.*, 2014).

To determine whether there is a stimulation method that could yield a higher number of competent oocytes, one first needs to consider the effects of LH and FSH as the principal drivers of ovarian stimulation, and their pharmacodynamics. In a natural cycle, FSH receptor expression peaks during the early follicular phase then declines, while LH receptor expression increases from mid-follicular phase, indicating that LH is likely to be involved in follicular development (Jeppesen *et al.*, 2012). This could explain, at least in part, why follicular recruitment is compromised in women with profound pituitary downregulation (Ferraretti *et al.*, 2004). The role of the theca cells in ovarian responsiveness to FSH is also illustrated by compromised follicular recruitment in women older than 35 years (Piltonen *et al.*, 2003; Humaidan *et al.*, 2004; Hugues *et al.*, 2010), particularly those with reduced ovarian sensitivity to FSH (Davison *et al.*, 2005) and reduced ovarian capacity to secrete androgens under basal LH conditions (Spencer *et al.*, 2007). In addition, it has been shown that LH induces epidermal growth factor-like factors in the mural granulosa. Amphiregulin, one of these factors, has been correlated with good oocyte quality (Zamah *et al.*, 2010; Sugimura *et al.*, 2015).

The time of oocyte retrieval relative to the time of the ovulation trigger is typically in the range of 34–38 h. In a meta-analysis including five RCTS with a total of 895 women, it was found that a time of oocyte retrieval relative to the time of the ovulation trigger of >36 h compared to <36 h resulted in a higher oocyte maturation rate, but no difference in fertilization rate, implantation rate or pregnancy rate (Wang *et al.*, 2011). Deviations from the locally established protocol should be documented and taken into consideration.

In conclusion, to optimize outcomes, there is a need for individualization of the treatment protocol. As stimulation can affect a range of systems, closely monitoring the patient's response could reduce many of the risks associated with ovarian stimulation, as stimulation can affect a range of systems, which will also have an impact on the success of treatment.

Because of the interlinked effects of ovarian stimulation on oocyte quality and developmental competence, it is logical that the most successful clinics are those in which the embryologists and clinicians speak with each other and communicate regularly and effectively about outcomes related to stimulation (and other procedures) (Van Voorhis et al., 2010).

Oocytes

Not all oocytes collected from a patient following ovarian stimulation for ART will have the same developmental competence, which is illustrated by the observation that only 5% of oocytes collected eventually result in a live birth (Lemmen et al., 2016). Intrinsic oocyte competence is derived not only from the degree of nuclear maturity of the oocytes, but also from their cytoplasmic maturity (Patrizio and Sakkas, 2009; Garrido et al., 2011; Lemmen et al., 2016). Furthermore, oocyte developmental competence is affected by a range of intrinsic patient-related and external factors. These patient-related factors include age, BMI, lifestyle factors and type of infertility. The external factors include ovarian stimulation, laboratory procedures (such as oocyte retrieval, denudation, cryopreservation, preparation for ICSI), culture conditions (temperature, pH, pO_2), environmental conditions (light, air quality, humidity) and culture medium.

Against that background, the question is whether any laboratory indicator can provide a measure of the intrinsic oocyte competence at the time of oocyte retrieval, as all of the subsequent events could be influenced by laboratory procedures, and/or by the genetic contribution of the spermatozoon. In other words, is quality measurable for oocytes, or perhaps more pertinently, is there any measure that could pinpoint where a dysfunction occurred during the long process of oocyte development?

There are a number of possible markers for oocyte competence, but these are largely research-based, and have not found widespread application in clinical service. These include assessment of biochemical markers in follicular fluid, gene expression studies of follicular cells and oxygen uptake assessments (Nagy et al., 2009; Nel-Themaat and Nagy, 2011). Other markers, such as assessment of oocyte morphology, spindle imaging, and polar body (PB) biopsy, can be incorporated into clinical service, but this is not a universal approach (Patrizio et al., 2007; Rienzi et al., 2011; Braga et al., 2013).

When the results of the Alpha and ESHRE surveys were combined, respondents identified oocyte recovery rate and oocyte maturity rate as the most important indicators for oocytes. Although, strictly speaking, they do not provide an indication of laboratory performance, they do provide an estimate of response to stimulation, and therefore a general estimation of the likely developmental competence of the oocytes.

In the Alpha survey, oocyte recovery rate was defined as the likelihood of aspirating a COC from each follicle over a certain size as measured on the day of triggering. The rationale for this is the expectation that those follicles that have achieved a certain size, with a good response to FSH and a sufficient number of LH receptors in follicular cells, will respond appropriately to the ovulation signaling cascade, resulting in the release of the COC into the follicular fluid, thereby facilitating its aspiration. A concern with this potential indicator was its reliance on the accuracy of follicular scanning, and the need for a consistent time interval between ovulation trigger and oocyte retrieval. However, while a range of follicle sizes was identified in the survey as the 'ideal' size for triggering, the expected recovery rates were remarkably similar, generally ranging from 70 to 80% as the competence level, and 85–100% as the benchmark value.

Oocyte maturity rate is generally related to nuclear maturity, being defined as the proportion of oocytes at MII stage. Its potential value is as a marker of the efficiency of ovarian stimulation and triggering. Of the Alpha survey respondents, 80% indicated that their laboratory determined the MII rate, with median competence and benchmark values of 75 and 90%, respectively. It was noted that the timing of this assessment is an important factor, as it is not possible to assess oocyte maturity at the time of oocyte retrieval in the case of insemination by routine IVF. Since this assessment requires the removal of the cumulus and corona cells, it can be performed at the time of denudation of the oocytes prior to ICSI, but for a universal competence and benchmark value to be established, a consistent time interval between the time of trigger and the time of cumulus cell removal would be required (e.g. 40 ± 1 h post ovulation triggering).

A third potential indicator, oocyte grade, was defined as the proportion of COCs with expanded cumulus at the time of oocyte retrieval. As ovulation triggers cumulus expansion by mediating the synthesis of hyaluronic acid and the organization of a stable cellular matrix (Russell and Salustri, 2006), this indicator provides an assessment of the quality of communication between the oocyte and its cumulus cells. The potential problems associated with the use of this indicator are a lack of objective criteria for making this assessment, and a concern that there is not always a good correlation between nuclear maturity and cumulus cell expansion (Balaban and Urman, 2006).

Other indicators that were proposed but not considered valuable or reliable by the survey respondents were: rate of degenerated (or empty) zonae pellucidae; rate of germinal vesicle oocyte recovery; definition of the minimum number of follicles to justify flushing; and oocyte degeneration rate at the time of oocyte retrieval. Oocyte degeneration rate after removal of cumulus cells is discussed in the section on ICSI.

Overall, the responses to the surveys highlighted the lack of consistent data for the evaluation of oocyte quality and competence, and identified an opportunity for national and international registries to promote the collection of this information.

Sperm KPIs

Proposed andrology laboratory PIs were sperm recovery rate, and sperm motility post-wash. In addition, sperm parameters were discussed in relation to the decision for IUI, IVF or ICSI.

Survey responses revealed such wide ranges in perceived semen analysis minima for suitability for IUI, IVF or ICSI, as well as expected sperm recovery post-wash, as to make it impossible to determine robust recommendations for competency and benchmark values for any of these criteria.

With regard to sperm preparation, it is possible that respondents were confused when reporting the 'recovery rate', and the substantial variability in terms of the expected/required number of spermatozoa in the final preparation likely included confusion between percentage recovery ('yield') values and the actual number of spermatozoa (millions); hence these data were considered unreliable. However, the expected proportion of motile spermatozoa in the final washed preparation showed coherence across the respondents, with both median and mode values of 90% for competency and 95% for the benchmark.

A major issue when considering semen analysis data is that many ART laboratories do not employ methods that meet the minimum standards required by either the World Health Organization (WHO) or the ESHRE SIG Andrology (Björndahl *et al.*, 2010; World Health Organization, 2010). Therefore, reported values for sperm concentration and motility must be understood to have high uncertainty of measurement, and hence need to be considered as inaccurate and unreliable (Sanchez-Pozo *et al.*, 2013; Björndahl *et al.*, 2016). As a consequence, any association between semen analysis characteristics, yield and fertility potential will remain unclear if based on studies using inappropriate semen analysis techniques. From a best practice standpoint, any clinical laboratory providing semen analysis or post-preparation values that are to be used for diagnostic or treatment management purposes should participate in an EQA programme which provides a comparison between the participating laboratories' results and established reference ('correct answer') values so as to permit quality improvement in laboratory work (Björndahl *et al.*, 2010).

Moreover, there is a general concern that semen analysis reference values have little or no value for ART procedures (Björndahl, 2011). In particular, the WHO reference values for sperm concentration, motility and vitality were derived from populations of men who had achieved *in vivo* conceptions (Cooper *et al.*, 2010), and therefore these cut-off values have no *a priori* relevance in regard to ART patients, and hence the need or suitability for any form of ART treatment should not be decided based on these reference values.

The Tygerberg Strict Criteria for normal sperm morphology were derived in regard to ART success (Kruger *et al.*, 1988; Coetzee *et al.*, 1998), so these cut-offs might be pertinent in differentiating between the need for IUI, IVF or ICSI—although concern regarding measurement uncertainty cannot be ignored (Menkveld *et al.*, 2011). While a cut-off of 4% normal forms might help to define sub-populations of patients with differing prognoses, at the level of individual patients a result of 4% based on 200 spermatozoa evaluated is not very informative since the result has a statistical expectation ranging from 2 to 8% (Björndahl *et al.*, 2010), and to be able to differentiate between 3 and 5% with statistical robustness would require the assessment to have been made by evaluating over 1500 spermatozoa.

Because of the limitations of semen analysis cut-off values, a decision on the suitability of IUI or routine IVF for a couple should be made based on post-preparation sperm numbers/concentration and their motility, ideally assessed during a pre-treatment 'trial wash' (while still taking into account the uncertainty of measurement). In case of ICSI, there should not be any cut-off based on semen analysis characteristics, the only logical criterion would be having sufficient (in comparison with the expected number of oocytes) spermatozoa that are, ideally, viable, and preferably motile or positive in the hypo-osmotic swelling (HOS) test (Nagy *et al.*, 1995). As a general principle, laboratories should develop and apply their own criteria for deciding on IUI, IVF or ICSI, based on the couple's clinical situation and reproductive history rather than semen analysis.

Regarding andrology laboratory PIs, results from the Alpha survey indicated that only post-preparation sperm motility would be a valuable indicator, as it monitors the effectiveness of the sperm washing procedure. Therefore, post-wash sperm motility should be monitored for fresh ejaculate specimens that show normozoospermia as per the WHO5 guidelines (World Health Organization, 2010), but still taking into account the poor reliability of sperm motility data, non-robust classification of semen samples based on the high uncertainty of semen analysis data, and variability in sperm preparation methods.

Sperm recovery rate, defined as the percentage recovery of progressively motile sperm after washing as compared to pre-washing (Björndahl *et al.*, 2010), can be used as a laboratory KPI, providing useful information for inter-operator comparison and proficiency testing. However, given the high uncertainty in counting and the different protocols for sperm preparation (notably with density gradient washing resulting in higher recovery rates as compared to direct swim-up from semen), no competence values can be provided. Laboratories should develop their own standards according to their own clinical and laboratory practice.

Fertilization after insemination by ICSI

Although several potential KPIs have been identified in ICSI, the presentation focused on the four most pertinent, i.e. normal fertilization rate, oocyte degeneration rate, poor fertilization rate and failed fertilization rate.

The definition used most often for the ICSI normal fertilization rate is the proportion of injected oocytes with 2PN the day after injection, except for the Spanish Registry and the Istanbul Consensus which include the observation of two PBs in the definition (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011). The suggested competence and benchmark values for this indicator were consistent among respondents ranging from 60 to 80%, and 70 to 100%, respectively. The UK's Association of Clinical Embryologists proposed benchmark for the 2PN rate is >65% including only patients below 40 years of age with at least three oocytes collected (Hughes and Association of Clinical Embryologists, 2012). From the literature, it was found that ICSI results in an average fertilization rate of 70% (Heindryckx *et al.*, 2005; Kashir *et al.*, 2010), which was similar to 68.7% based on over 20 000 unselected MII oocytes at the CARE Fertility laboratory, UK (personal communication Alison Campbell). ICSI fertilization rate is a commonly reported and effective indicator that is informative of gamete quality and operator competence. ICSI 2PN rate does depend on the various criteria used for performing ICSI, which can be considered a weakness of the indicator.

The ICSI damage rate or oocyte degeneration rate was ranked as important. In the Alpha survey, the minimum expected value and target value ranged from 3 to 30% and 0 to 10%, respectively. Oocyte damage can be observed at three time points during the ICSI process: from the start at stripping, during ICSI, or at the fertilization assessment on Day 1. Oocyte damage probably occurs most frequently during injection, but without immediate signs of damage, this is not detected until the fertilization check. In addition, as both the damage detected at injection and at fertilization check reflect damage from the ICSI process, these should not be recorded and calculated separately. Damage at denudation/stripping can be monitored separately as it mainly reflects operator's competency, but it has a very low frequency. ICSI damage rate is therefore defined as the number of oocytes damaged during ICSI, and/or observed at fertilization check over the number of injected oocytes. It is useful to monitor this indicator for operator competence, oocyte quality, and laboratory performance. The damage rate can also be indicative of technical problems (e.g. cumulus cell removal stress, vibration). Alternatively, the term ICSI oocyte survival rate can be used.

Poor fertilization rate is defined as the proportion of cycles in which <25% of the injected oocytes are fertilized. The responses from the

survey are much divided, ranging from 5 to 20% for the competence and from 0 to 15% for the benchmark value. Poor fertilization rate can give an indication of operator competence and reflect gamete quality.

Failed fertilization rate scored relatively low on importance in the surveys. Failed fertilization rate is defined as the proportion of cycles in which none of the injected oocytes are fertilized. The indicator can be informative of gamete quality/function and/or operator skill. A deficiency in the mechanism of oocyte activation is regarded as the principal cause of ICSI fertilization failure or abnormally low fertilization. Complete ('or virtually complete') fertilization failure with ICSI occurs in 1–5% of cycles (Liu *et al.*, 1995; Flaherty *et al.*, 1998; Mahutte and Arici, 2003; Yanagida, 2004; Kashir *et al.*, 2010). From the Alpha survey there was a wide range in the competence values for this indicator ranging from 2 to 15% (median 5%), with a benchmark of <1%.

For these and other KPIs, a reference population could be relevant. With regard to ICSI fertilization rate, it could be relevant to exclude cases where reduced fertilization rates are anticipated, including *in vitro* matured metaphase I oocytes (although inconclusive data), artificially activated oocytes, the use of testicular sperm, and cases of globozoospermia and asthenozoospermia (Rubino *et al.*, 2016). However, owing to the low prevalence, including these cases may not significantly affect the indicators in most clinics.

In conclusion, from the surveys and collected evidence, ICSI damage rate and ICSI normal fertilization rate are considered relevant and important KPIs, while the value of ICSI low/failed fertilization rate as a KPI is less clear. Oocyte maturity rate and 1PN/3PN rate were not specifically discussed for ICSI. The ICSI rate, defined as the proportion of cycles that use ICSI, was not commonly recorded by the survey respondents, judging this as a less relevant PI.

Fertilization after (routine) IVF insemination

This section deals with normal fertilization rate, polyspermy rate, poor fertilization rate and zygote morphology after routine IVF insemination.

Pronuclear formation occurs 1.5–2.0 h earlier in oocytes inseminated by ICSI compared with those inseminated by conventional IVF (Nagy *et al.*, 1998; Montag *et al.*, 2001). This should be taken into consideration when setting the time for fertilization check, relative to the time elapsed since insemination (recommended as 17 ± 1 h) (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011). A normally fertilized oocyte should have 2PN of similar size that are closely apposed and centrally located (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011). Although the provenance of micronuclei remains unclear, their presence could be considered to be abnormal, as could the presence of pronuclei of different sizes. From the literature, the normal fertilization rate (i.e. the proportion of inseminated oocytes with 2PN at the time of the fertilization check on Day 1) is expected to be around 67%, with a range of reported values of 53–81%. This range was reflected in the Alpha survey results, where the median value for competency was 61% of inseminated oocytes, with a median benchmark value of 77%.

The presence of three or more pronuclei is indicative of an abnormal fertilization, arising from either nondisjunction (failure to extrude the second PB) or polyspermy. Polyspermy may be the result of either oocyte immaturity (causing failure of the cortical reaction), oocyte overmaturity and/or an extremely high concentration of motile spermatozoa in the insemination volume (Wang *et al.*, 2003). From the literature, the incidence of ≥3PN is 4–7% in IVF (Joergensen *et al.*, 2015). This agrees well with the median values from the Alpha survey of <9% for competence, and <4.5% as a benchmark.

The calculation of the total fertilization rate following IVF includes all fertilized oocytes with ≥2PN. Although, as already stated, oocytes with >2PN are abnormally fertilized, this parameter provides an indication of the ability of the culture system to support sperm capacitation and sperm-oocyte interaction in IVF cycles. Of the published studies that included >100 oocytes, the median total fertilization rate was 76% (range 69–87%) in selected patient populations. This is similar to the Alpha survey results, which suggested a competency level of at least 63% and a benchmark of at least 84%.

Oocytes with a single pronucleus after insemination by IVF, which occurs in 1–5% of cases, can be indicative of fertilization and syngamy, asynchronous appearance of pronuclei (an extremely rare event, as evidenced by the use of time-lapse microscopy), or parthenogenetic activation (Levron *et al.*, 1995). The incidence of diploidy in 1PN oocytes following conventional IVF has been reported to be in the range of 45–50% (Sultan *et al.*, 1995; Staessen and Van Steirteghem, 1997; Kai *et al.*, 2015). In contrast, 1PN oocytes arising after ICSI have a reported diploidy rate of only 7–14%, with genetic abnormalities in the subsequent embryos (Mateo *et al.*, 2013).

The incidence of poor fertilization (<25% of inseminated COCs with 2PN) or total failure of fertilization (no oocytes with signs of fertilization) could be indicative of a problem with sperm function, too few motile spermatozoa during insemination, or failure of oocyte activation (Ebner *et al.*, 2015). There is very little evidence in the literature regarding the expected incidence of either poor or failed fertilization. However, the Alpha survey results suggested competency and benchmark levels for poor and failed fertilization of 14 and 6%, and 8 and 4%, respectively.

Although the Istanbul Consensus made recommendations about grading zygote morphology (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011), the question remains as to whether indicators referring to zygote morphology are useful, especially as differences in pronuclear pattern could be related to the insemination method and timing of the observation (Montag *et al.*, 2001; Ebner *et al.*, 2003).

Cleavage-stage embryos

Proposed indicators for cleavage-stage embryos are early cleavage rate, cleavage rate, embryo development rates, embryo fragmentation rate, and rate of good quality embryos (embryo score or grade).

Early cleavage rate is defined as the proportion of cleaved zygotes at the early cleavage check on Day 1 (26 ± 1 h post-ICSI or 28 ± 1 h post-IVF) (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011), but other time-intervals after insemination have been used for assessing early cleavage. This indicator reflects the ability of the culture system to support early cleavage of fertilized oocytes and the viability and quality of the embryos (Shoukir *et al.*, 1997). There are conflicting results on the importance of early cleavage. Studies have shown that early cleavage, together with other factors, can be used as an embryo selection method (Lundqvist *et al.*, 2001; Ciray *et al.*, 2005). Early cleavage rate has also been shown to correlate with blastocyst implantation and pregnancy rates (Shoukir *et al.*, 1997; Balaban and Urman, 2003) and it is a better independent marker of implantation

potential than zygote morphology (Brezinova *et al.*, 2009). In contrast, early cleavage was not found to be an independent predictor of implantation in IVF patients with good prognosis (Thurin *et al.*, 2005). In addition, premature occurrence of early cleavage can be negatively, instead of positively, associated with embryo implantation potential (Meseguer *et al.*, 2011). Furthermore, it was not a reliable predictor for embryo implantation rate when good quality embryos are transferred (Sundstrom and Saldeen, 2008; de los Santos *et al.*, 2014), or when using a GnRH antagonist protocol (Yang *et al.*, 2015). In the Alpha survey, competence and benchmark values ranged from 10 to 50% and 15 to 60%, respectively.

Cleavage rate reflects the ability of the culture system to support cellular division of fertilized oocytes. It is an indicator of embryo viability and has the ability to detect culture media contaminants. The presence of non-cleaved embryos or arrested zygotes on Day 3 is associated with a decrease in quality of the remaining cohort, but without a negative impact on clinical outcome (Machtinger *et al.*, 2015). Cleavage rate is considered important and is widely monitored, and defined as the proportion of zygotes which cleave to become embryos on Day 2 at 44 ± 1 h post-insemination (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011). Cleavage rate should be calculated not only in the total population, but also in reference groups (IVF versus ICSI, female age, ejaculated versus surgically retrieved sperm), and controlled for confounders (the timing of observation and oocyte maturity). Also, the presence of a refractile body in the oocyte is associated with reduced cleavage rates and impaired embryo development, while the cytoplasmic granularity did not seem to have an effect on embryo development (Fancsovits *et al.*, 2012). Cleavage rate should be calculated frequently in a laboratory (at least once per month). In the Alpha survey, the competence values ranged from 80 to 95%, with a benchmark of 90 to 100%.

Embryo development rate is defined as the proportion of 4-cell embryos on Day 2 among the 2PN zygotes (measured at 44 ± 1 h post-insemination), the proportion of 8-cell embryos on Day 3 (measured at 68 ± 1 h post-insemination), and the proportion of morula-stage embryos on Day 4 (92 ± 2 h post-insemination) (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011). This indicator reflects the ability of the culture system to support cleavage according to the expected developmental stages and the quality and viability of embryos, especially for Day 2 or 3 transfer, while less important for blastocyst transfer. Possible confounders are the timing of laboratory observations and the type of culture media used. Although dependent on iatrogenic factors such as the culture conditions, embryo development rate is an important indicator; in well-defined categories of patients, it reflects the overall laboratory performance. It was stated that sufficient numbers of embryos or longer data collection are necessary, as this indicator is influenced by short-term variations. Calculation of a sliding mean can be helpful for detecting long-term variations. The value and practicability of the morula check on Day 4 was questioned as centers performing Day 5 embryo transfer often do not assess the embryo development at Day 4. Also, instead of assessing the number of 4-cell embryos on Day 2, or 8-cell embryos on Day 3, a combination of 4-cell and 8-cell embryos on Days 2 and 3 can be used. This was analyzed in a study by van Royen, who thereby characterized a top quality embryo as having 4- to 5-cells on Day 2 and ≥ 7 cells on Day 3 (Van Royen *et al.*, 1999). In larger centers, assessing the embryos at the specified time points can be difficult, and counting 4- and 5-cell embryos on Day 2, and 7-, 8- and 9-cell embryos on Day 3 may be more relevant.

The rate of good quality embryos is defined as the proportion of Days 2 and 3 embryos with high score or grade. Many different scoring systems exist, based on different variables, including cell number, fragmentation, cell size and multinucleation (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011) and blastomere nuclear status (Fauque *et al.*, 2013). A recent study evaluating which set of embryo variables is most predictive for live birth rate (LBR) reported that blastomere number, proportion of mononucleated blastomeres, degree of fragmentation and variation in blastomere size were significantly associated with LBR in univariate analysis, while symmetry of the embryo was not (Rhenman *et al.*, 2015). Furthermore, the grading systems are not robust, but can be used as internal quality assessment parameters. The importance of this parameter is also affected by the limited significance of the fragmentation rate.

Embryo fragmentation rate, defined as the proportion of Days 2 and 3 embryos with <10% fragmentation, reflects the quality and viability of embryos. From the Alpha survey, the competence value for this parameter ranged from 20 to 90% (median 50%) and the benchmark from 30 to 90% (median 70%). These large ranges underline the difficulties with this parameter: embryo fragmentation rate is reported to be a subjective parameter and difficult to evaluate as one has to differentiate between a cell and a fragment and then estimate the relative proportion of fragments (Paternot *et al.*, 2011).

Embryo utilization rate is defined as the number of embryos utilized (transferred or cryopreserved) per number of 2PN zygotes in the same cycle. This parameter is often presented in studies, but competence and benchmark values cannot be calculated because of its dependence on strategies for embryo transfer and cryopreservation, as well as patient request.

In conclusion, embryo cleavage rate and embryo development rate are extremely important indicators, while early cleavage rate, rate of good quality embryos and embryo fragmentation rate are less important as quality indicators.

Blastocyst development

In the case of blastocyst-stage embryo transfer, several parameters were suggested as indicators: blastocyst development rate, good blastocyst development rate, the proportion of good quality blastocyst and Day 5 embryo transfer rate.

The blastocyst development rate, defined as the proportion of 2PN zygotes (not just of cleaved zygotes) which are at the blastocyst-stage at Day 5 (116 ± 2 h post-insemination) (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011), was rated important because it reflects the efficiency of the whole culture system. Blastocyst development rates can be calculated on Day 5, 6 or Days 5/6 combined. Assessment and calculation on Day 5, consistent with previous consensus, is preferred based on limited numbers of embryos available on Day 6. The competence and benchmark values for blastocyst development rate on Day 5 ranged from 25 to 60%and 44 to 80%, respectively. The blastocyst development rate is an objective parameter, but dependent on the assessment of blastocyst morphology, which is straightforward in case of good quality blastocysts, but can be challenging for embryos showing an attempt of cavitation. Confounders can be the timing of laboratory observation, the culture medium and the culture conditions (in particular the pO_2 concentration).

A good blastocyst development rate is defined as the proportion of 2PN zygotes which are good quality blastocysts on Day 5 (116 ± 2 h post-insemination) (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011). Similar to blastocyst development rate, measuring this as an indicator only on Day 5 could make the indicator more robust. Blastocyst quality should be based on three factors, namely blastocoele expansion, appearance of trophectoderm (TE) and appearance of inner cell mass (ICM). Although all three parameters have been shown to be significantly correlated to pregnancy and LBR (Van den Abbeel *et al.*, 2013), only TE was found to be a statistically significant independent predictor of live birth outcome after adjustment for known confounders (Ahlstrom *et al.*, 2011). Even though the ICM is important, a strong TE layer is essential at this stage of embryo development, allowing successful hatching and implantation (Ahlstrom *et al.*, 2011). In the Alpha survey, the competence and benchmark values for good blastocyst development rate on Day 5 ranged from 15 to 45% and 25 to 80%, respectively.

The proportion of good quality blastocysts can be calculated from the blastocyst development rates and good blastocyst development rates. There is no evidence pertaining to the significance of this parameter in the literature, and no data from the Alpha survey.

Day 5 embryo transfer rate was defined as the proportion of cycles with ≥1 2PN zygotes on Day 1 that had ≥1 blastocyst for transfer on Day 5. From the Alpha survey there was a large variation in the values for competence and benchmarks (ranging from 25 to 90% and 40 to 100%, respectively), which is assumed to be related to differences in the time of blastocyst assessment, and different grade of expansion. In addition, this parameter depends on different policies for transfer in different settings. PGD/PGS (globally indicated as preimplantation genetic testing—PGT—that includes PGD for single gene disorders or for chromosome structural abnormalities, and PGS for aneuploidy) cycles should be excluded from this calculation. Some possible confounders are the timing of laboratory observation (ideally at 116 ± 2 h post-insemination), but in some laboratories the time of observation depends on the timing of embryo transfer (physician availability), the culture medium and other culture conditions (e.g. pO_2 concentration), and the grade of blastocyst expansion.

Fresh cleavage or fresh blastocyst transfer?

Recently, a Cochrane review summarized evidence from RCTs comparing the reproductive outcomes after fresh cleavage-stage versus fresh blastocyst-stage embryo transfer (Glujovsky *et al.*, 2016). Based on low-quality evidence, they concluded that the LBR following fresh transfer was higher in the blastocyst transfer group as compared to the cleavage-stage transfer group [odds ratio (OR) 1.48, 95% CI 1.20–1.82; 13 RCTs, 1630 women, $I^2 = 45\%$]. This is translated in a LBR of 29% after fresh cleavage-stage transfer, and between 32 and 42% after fresh blastocyst-stage transfer. The five RCTs that reported cumulative pregnancy rates after fresh and frozen transfers, showed no significant difference after one round of oocyte retrieval (based on very low-quality evidence). The main limitation was serious risk of bias, associated with failure to describe acceptable methods of randomization, and unclear or high risk of attrition bias (Glujovsky *et al.*, 2016). Another review reported that blastocyst (Day 5/6) transfer in a fresh IVF/ICSI treatment cycle significantly increased LBR (OR 1.77; 95% CI 1.32–2.37), clinical pregnancy rate, implantation rate, and ongoing pregnancy rate, and reduced first trimester miscarriage rate, in comparison with cleavage-stage (Day 2/3) embryos transfer based on seven RCTs and 1446 cases (Wang and Sun, 2014).

Implantation rate and LBR

Implantation rate is judged an important indicator that reflects the overall performance of the laboratory and an overall low implantation rate is a serious alert. Implantation rate is defined as the number of gestational sacs observed divided by the number of embryos (cleavage-stage or blastocysts) transferred (Zegers-Hochschild *et al.*, 2009), or as the proportion of fetal hearts relative to the number of embryos transferred (Alpha Scientists In Reproductive Medicine, 2012). Implantation rate reflects the efficiency of the whole culture system, but it can be influenced by uterine receptivity, and by different policies for embryo transfer in different centers.

LBR may be considered as the ultimate KPI for checking IVF clinic performance and defined as the likelihood of a baby to be born per embryo transferred. LBR is largely affected by a series of clinical maternal factors pertaining to post-implantation development, rather than reflecting laboratory performance. This parameter can be calculated annually, but it is often difficult to collect the data.

PGT: PGD/PGS

The indicators proposed for PGD/PGS (PGT) were successful biopsy rate/tubing rate, rate of no biopsy, proportion of samples submitted to analysis where no results were available (no DNA was detected), and embryos not found on warming. Other indicators suggested in the survey were 'survival after warming', embryo transfer per PGT cycle, and miscarriage rate, but there were very few responses for these variables in the Alpha survey.

A successful biopsy rate is defined as the proportion of biopsied and tubed/fixed samples where DNA is detected. It was suggested to use this parameter combining the tubing rate (the proportion of embryos where cells were tubed) and the proportion of samples submitted to analysis where no DNA was detected, as the tubing of the cells can often not be inspected visually and will only be detected by the presence of DNA after amplification. A benchmark for the tubing rate of 95% was reported in the Alpha survey. Confounders for the successful biopsy rate are embryo quality, and the criteria for biopsy (for Day three embryos: presence of a visible nucleus; for Day 5/6: grade of hatching, TE quality). In the data of the ESHRE PGD Consortium, of 254 820 samples that were biopsied, 91.3% were diagnosed (De Rycke *et al.*, 2015). It is important to underline that, as already mentioned, these data mainly refer to Day 3 biopsies and that in the case of PGT for aneuploidy (generally known as PGS) the analysis of biopsies was mainly by fluorescence *in situ* hybridization (FISH), a technique requiring cells to be fixed on a glass slide. FISH is now being replaced by other methods providing 24-chromosome testing, which require tubing of cell biopsies.

The rate of no biopsy is defined as the proportion of intended PGD/PGS (PGT) cases where there were no embryos available to biopsy. This parameter was rated important, but it reflects patient-related factors and the ability of the culture system to support cleavage/blastocyst formation, rather than the performance ability of the laboratory to perform a PGD/PGS (PGT) treatment/analysis. Furthermore, the parameter is different whether biopsy is performed at the cleavage or blastocyst-stage, and depends on the timing of the

laboratory observation, culture medium and culture conditions (e.g. pO_2 concentration), criteria for biopsy, the time point of assisted hatching (Day 3 or 5), and patient selection. The parameter should also be calculated for PGD and PGS separately, based on the difference in patient populations. From the Alpha survey, the median competence value and benchmark were 20 and 10%, respectively. In the last data collection of the ESHRE PGD Consortium, out of 45163 reported cycles, 2.8% were canceled before biopsy (De Rycke *et al.*, 2015). However, as this data collection covers cycles performed up to 2010, this figure refers to Day 3 biopsies, implying that possibly a higher value could be expected for Day 5 biopsies.

For a number of samples, no results are available after amplification. Results can be inconclusive even with a strong DNA amplification band in the agarose gel, which can be due to early fragmentation of DNA. In single gene analysis, results can be inconclusive if information is found only on a reduced number of markers, or the gene of interest failed to amplify. Although this indicator is not strictly related to the performance of the laboratory, it is relevant to inform clinicians on the total number of embryos where PGD/PGS (PGT) results are available. The number of samples where no results are available after amplification was reported in the Alpha survey with a benchmark of <5%, which corresponds with the converse of the tubing rate.

Nowadays, the majority of PGT cycles are based on blastocyst biopsy and cryopreservation. The proportion of embryos not found on warming and embryos degenerated after warming mainly reflects operator skills. In the Alpha survey, the median competence values were 3 and 10% for not found and degenerated embryos, respectively. These indicators can also be reported as one combined indicator. It was mentioned that clinicians should be aware that although laboratories strive for 100%, not all embryos submitted to PGD/PGS (PGT) will be recovered after warming.

Reference values for implantation rate and LBR after PGT can be derived from the data from the ESHRE PGD Consortium. The implantation rate was 26% for PGD and 22% in PGS, with delivery rates (per oocyte retrieval) of 21 and 14%, respectively (De Rycke *et al.*, 2015).

Indicators for cryopreservation: addition to the previous consensus

Blastocyst re-expansion rate is defined as the proportion of warmed blastocysts that show re-expansion within a defined time period (e.g. 2 h) (Alpha scientists in reproductive medicine, 2012). Recent evidence shows an impact on the performance results depending on the quality/expansion of the blastocysts which are cryopreserved (Cobo *et al.*, 2012). Also, in blastocyst fresh transfer, from multivariate analysis it was shown that the odds of live birth increased by 36% for each grade of expansion ($P = 0.0061$) and decreased by 29% for blastocysts with grade B TE compared with Grade A TE ($P = 0.0099$). Furthermore, after thawing, the odds of live birth increased by 39% ($P = 0.0042$) for each 10% increase in degree of re-expansion. Therefore, blastocoel expansion and TE grade were selected as the most significant pre-freeze morphological predictors of live birth and degree of re-expansion was selected as the best post-thaw parameter for prediction of live birth (Ahlstrom *et al.*, 2013). Confounding factors are time of observation, female age and fertilization method. These observations do not include embryos that had been biopsied on Day 3 as they have a different hatching dynamic (Lopes *et al.*, 2015).

Recommendations of the expert panel

General comments

(1) Regarding frequency of data collection for indicators, it was the consensus opinion that this should be done, ideally, on a monthly basis. However, it was recognized that this is not always practical, based on caseload, and therefore either a longer timeframe or a specific predetermined number of cases might be used instead. The minimum number will depend on the stability of the indicator and will need to be developed by the laboratory, although an initial dataset of 30 cases could be used as a guideline. Nonetheless laboratories should remain vigilant and respond promptly to unexpected fluctuations.

(2) The discussions identified three different types of indicators: reference indicators (RIs), PIs and KPIs.
- RIs were related to the oocytes coming into the laboratory, and so were proxy indicators of the response to ovarian stimulation.
- PIs were those for which data should be documented and stored, even if they are not routinely reported in a control chart.
- KPIs were those related to the 'core business' of the ART laboratory.

(3) The values for indicators are presented as competency and benchmark values, as was done for the cryopreservation consensus (Alpha Scientists In Reproductive Medicine, 2012). The gap between the competency and the benchmark values is the 'desirable range'.

(4) It was the opinion of the expert panel that ovarian stimulation can have an impact on the overall treatment cycle but is less likely to have an impact on any single laboratory PI.

(5) To apply the recommended values:
- The time of oocyte retrieval relative to the time of the ovulation trigger is typically in the range of 34–38 h (mostly 36 h). Deviations from the locally established protocol should be documented and taken into consideration.
- The timing of all observations should be made as recommended in the Istanbul consensus (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011).

(6) Individual clinics should decide whether it is more practical to subdivide their results into specific patient groups for KPI and PI determinations, based on their clinical practice. The indicator values presented here were derived relative to cycles that met the criteria for a 'reference population'. With the exception of indicators with specific qualifiers identified, these criteria were:
- Female patients <40 years old;
- own fresh oocytes;
- ejaculated spermatozoa (fresh or frozen);
- no PGD/PGS (PGT);
- all insemination methods (i.e. routine IVF and ICSI).

(7) It was the opinion of the panel that national and international registries should be encouraged to gather data that can be used for the derivation of KPI standard values.

(8) Any selection procedure in addition to embryo morphology, for example PGD/PGS (PGT) or time-lapse imaging, is not expected to increase the cumulative pregnancy/LBR, but in the case of PGS it may result in reduced time to pregnancy/live birth.

Indicators

The results of the discussions have been summarized for the majority of the indicators. The values for indicators have also been presented in

Table II RIs for identifying performance of the ART laboratory.

RI	Calculation	Benchmark ialue
Proportion of oocytes recovered (stimulated cycles)	$\frac{\text{no. oocytes retrieved}}{\text{no. follicles on day of trigger}} \times 100$	80–95% of follicles measured
Proportion of MII oocytes at ICSI	$\frac{\text{no. MII oocytes at ICSI}}{\text{no. COCs retrieved}} \times 100$	75–90%

MII, metaphase II; RI, reference indicators; COC, cumulus-oocyte complex.

Table III PIs for the ART laboratory.

PI	Calculation	Competency value (%)	Benchmark value (%)
Sperm motility post-preparation (for IVF and IUI)	$\frac{\text{progressively motile sperm}}{\text{all sperm counted}} \times 100$	90	≥95
IVF polyspermy rate	$\frac{\text{no. fertilized oocytes with} > 2\text{PN}}{\text{no. COCs inseminated}} \times 100$		<6
1 PN rate (IVF)	$\frac{\text{no. 1PN oocytes}}{\text{no. COCs inseminated}} \times 100$		<5
1 PN rate (ICSI)	$\frac{\text{no. 1PN oocytes}}{\text{no. MII oocytes injected}} \times 100$		<3
Good blastocyst development rate	$\frac{\text{no. good quality blastocysts on Day 5}}{\text{no. 2PN/2PB oocytes on Day 1}} \times 100$	≥30	≥40

PN, pronucleus; PI, performance indicator; PB, polar body.

Tables II–IV, but these should be read in association with the summary for each indicator.

Proportion of oocytes recovered

The proportion of oocytes recovered RI (Table II) is defined as the number of oocytes retrieved as a function of the number of ovarian follicles seen at ultrasound assessment. It is useful as a measure of whether the quantity of oocytes is maximized. The values are not influenced by laboratory practice, and so cannot be held to be laboratory PIs, but values outside the expected range could prompt an investigation of any changes in stimulation practice. Having this information is an important factor in troubleshooting. The expected range is 80–95% of follicles measured in stimulated cycles.

Proportion of MII oocytes at ICSI

The proportion of MII oocytes at ICSI RI (Table II) is defined as the proportion of oocytes that have nuclear maturity at the time of injection, and so acts as a proxy indication of the effectiveness of ovarian stimulation. It is not a laboratory PI, as values are not influenced by laboratory practice, but rather reflects factors that influence the competence of oocytes coming into the laboratory. The expected range is 75–90% at 40 ± 1 h post-trigger for all COCs retrieved. Values outside this range could prompt a review of any changes in ovarian stimulation, triggering or follicle aspiration practice, as changes in the proportion of MII oocytes could be a factor in changes in fertilization rates and/or embryo development. Instability in this value could indicate changes in the stimulation, resulting in a higher proportion of either immature or post-mature oocytes.

Notes:

- Since this value is expected to be stable, laboratories may choose 'reporting by exception'—only reporting on it when it falls outside the expected range.
- It should be noted that nuclear maturity does not necessarily indicate cytoplasmic maturity of the oocyte (Sundstrom and Nilsson, 1988; Eppig, 1996; Coticchio *et al.*, 2012).
- Clinics should consider whether they should sub-divide their assessment of this indicator based on patient demographics.
- Good communication between laboratory and clinic (cycle planning and cycle review) were cited as being vital to excellent outcomes in IVF programmes (Van Voorhis *et al.*, 2010).

Semen analysis characteristics

Sperm concentration, motility and vitality. Unless semen analyses are performed employing analytical methods as per the ESHRE SIG Andrology (Björndahl *et al.*, 2010) or WHO5 (World Health Organization, 2010) the results for sperm concentration, motility and vitality will be subject to unacceptably high uncertainty of measurement (Sanchez-Pozo *et al.*, 2013; Björndahl *et al.*, 2016).

Sperm morphology. Sperm morphology assessment is subjective and so is dependent on consistent training. Since the Tygerberg Strict Criteria cut-off of 4% normal forms was derived in relation to IVF success, it could be pertinent in differentiating between the need for IUI, IVF or ICSI (Mortimer and Menkveld, 2001; Menkveld, 2010). However, the current visual evaluation of 200 or 400 spermatozoa used in the vast majority of laboratories to assess 'percentage normal forms' has such a large uncertainty of

Table IV KPIs for the ART laboratory.

KPI	Calculation	Competency value (%)	Benchmark value (%)
ICSI damage rate	$\frac{\text{no. damaged or degenerated}}{\text{all oocytes injected}} \times 100$	≤10	≤5
ICSI normal fertilization rate	$\frac{\text{no. oocytes with 2PN and 2PB}}{\text{no. MII oocytes injected}} \times 100$	≥65	≥80
IVF normal fertilization rate	$\frac{\text{no. oocytes with 2PN and 2PB}}{\text{no. COCs inseminated}} \times 100$	≥60	≥75
Failed fertilization rate (IVF)	$\frac{\text{no. cycles with no evidence of fertilization}}{\text{no. of stimulated IVF cycles}} \times 100$	<5	
Cleavage rate	$\frac{\text{no. cleaved embryos Day 2}}{\text{no. 2PN/2PB oocytes on Day 1}} \times 100$	≥95	≥99
Day 2 Embryo development rate	$\frac{\text{no. 4-cell embryos on Day 2}}{\text{no. normally fertilized oocytes}^a} \times 100$	≥50	≥80
Day 3 Embryo development rate	$\frac{\text{no. eight cell embryos on Day 3}}{\text{no. normally fertilized oocytes}^a} \times 100$	≥45	≥70
Blastocyst development rate	$\frac{\text{no. blastocysts Day 5}}{\text{no. normally fertilized oocytes}^a} \times 100$	≥40	≥60
Successful biopsy rate	$\frac{\text{no. biopsies with DNA detected}}{\text{no. biopsies performed}} \times 100$	≥90	≥95
Blastocyst cryosurvival rate	$\frac{\text{no. blastocysts appearing intact}}{\text{no. blastocysts warmed}} \times 100$	≥90	≥99
Implantation rate (cleavage-stage)[b]	$\frac{\text{no. sacs seen on ultrasound}^c}{\text{no. embryos transferred}} \times 100$	≥25	≥35
Implantation rate (blastocyst-stage)[b]	$\frac{\text{no. sacs seen on ultrasound}^c}{\text{no. blastocysts transferred}} \times 100$	≥35	≥60

[a]Defined as oocytes with 2PN and 2PB on Day 1.
[b]Based on total number of embryos transferred to *all* patients in the reference group, not just those for whom an implantation occurred.
[c]Definition reached after discussion, as some felt that no. fetal hearts / no. embryos transferred was a more meaningful indicator.
KPI, key performance indicator.

measurement that it cannot be considered a reliable predictor for IVF success/failure for individual men (Kvist and Bjorndahl, 2002; Björndahl *et al.*). Unless determined using a more robust methodology, sperm normal forms should not be used to direct ART treatment options.

Sperm motility post-preparation

The sperm motility post-preparation PI is defined as the proportion of progressively motile spermatozoa in the sperm preparation for insemination, and includes only fresh normozoospermic ejaculate specimens. Sperm motility after washing should be very high, and low values would indicate problems with the sperm preparation procedure. In this case, progressive motility is defined as spermatozoa that are moving with net space gain of the head, and so includes hyperactivated spermatozoa. Although there was excellent agreement among the survey responses, potential weaknesses of this PI include: possible poor reliability of sperm percentage motility data; non-robust classification of cases based on uncertainty of semen analysis data; variability in sperm preparation method used; and abnormal response of the sperm to the preparation method used. The reference values were competence 90% and benchmark ≥95%.

Notes:

– There is no sperm recovery rate KPI recommended because this is so heavily dependent on the processing method.
– *Recommendations for IUI or IVF treatment*:
It was the recommendation of the expert panel that decisions regarding a man's suitability or need for an appropriate ART treatment modality (IUI, IVF or ICSI) should be based on sperm numbers and motility assessments determined in a pre-treatment 'trial preparation'. The competency threshold value was agreed as at least 90% progressive motility post-wash with a benchmark of at least 95% progressive motility.
– *Recommendations for ICSI treatment:*
There was no cut-off value recommended for ICSI treatment, beyond the spermatozoa ideally being alive. In this case, the best evidence of vitality is motility, while the second-best evidence, in the absence of motility, is HOS-test positive (Björndahl *et al.*), although other methodologies exist.

ICSI damage rate

The ICSI Damage Rate KPI (Table IV) is defined as the proportion of oocytes that is damaged during the ICSI injection, or have degenerated by the time of fertilization assessment on Day 1. It is informative of gamete quality and/or operator skill, and excludes damage from oocyte stripping, which should be very rare. The results can be skewed by the patient mix or the stimulation protocols used, so all cycles should be included, to reduce the relative impact of these variables. The Alpha survey gave similar median and mode values for each level. These values were agreed by the expert panel in relation to those

recommended by the Association of Clinical Embryologists (Hughes and Association of Clinical Embryologists, 2012). The reference values for ICSI damage rate are: competence ≤10%; benchmark ≤5%.

ICSI normal fertilization rate

The ICSI Normal Fertilization Rate KPI (Table IV) is defined as the number of fertilized oocytes on Day 1 (presence of 2PN and 2PB assessed at 17 ± 1 h post-injection) (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011), as a function of all MII oocytes injected. This is a common, broad, effective indicator of good laboratory practice, as it is informative of gamete quality and/or operator skill. This KPI includes ejaculated spermatozoa only (fresh or frozen) as results may be lower with surgically retrieved spermatozoa, and excludes *in vitro* matured oocytes, as well as thawed/warmed oocytes (this was dealt with in the cryopreservation consensus) (Alpha Scientists In Reproductive Medicine, 2012). Reference values were agreed by the expert panel: competence ≥ 65%; benchmark ≥ 80%.

Notes:

- *Total ICSI failed fertilization rate:* It was the consensus of the expert panel that complete failure to achieve normal fertilization in an ICSI cycle did not need to be a PI, but should be reported by exception, meaning that 'every case' should be investigated. This includes only stimulated cycles, as natural cycles are expected to have only one oocyte.
- *Poor ICSI fertilization rate:* Although opinion regarding the expected incidence of cycles with ICSI fertilization rates <25% was sought in the Alpha survey, the consensus of the expert panel was to exclude this indicator, as it did not add to the information already collected.
- Giant oocytes should not be injected due to published evidence of chromosomal abnormality (Balakier *et al.*, 2002; Rosenbusch *et al.*, 2002; Lehner *et al.*, 2015).
- Regarding smooth endoplasmic reticulum (SER) positive oocytes, more recent publications of outcomes suggest that the Istanbul Consensus recommendation not to inject/inseminate these oocytes may need to be revisited (Mateizel *et al.*, 2013). It was the opinion of the expert panel that in the meantime, the decision to inject SER positive oocytes should be reviewed by the clinical team on a case-by-case basis. Follow-up of results, including pregnancy outcome and babies born, in case of insemination and transfer of the resulting embryos should be performed.

Normal IVF fertilization rate

The Normal IVF Fertilization Rate KPI (Table IV) is defined as the number of fertilized oocytes on Day 1 (presence of 2PN and 2PB assessed at 17 ± 1 h post-insemination (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011)), as a function of all COCs inseminated. IVF normal fertilization rate is an important indicator of laboratory performance, as it relies upon effective gamete handling and culture, and so is a measure of the whole IVF system. The reference values are: competency ≥60%; benchmark ≥75%. It should be noted that the benchmark value was determined based on a rounding of the product of the benchmark rates for MII oocytes (90%) and fertilization rate of MII oocytes (80%).

IVF polyspermy rate

IVF Polyspermy Rate PI (Table III) is defined as the proportion of inseminated oocytes with more than two pronuclei on Day 1 (17 ± 1 h post-insemination) and is needed to provide the information to interpret any observed variations in the normal fertilization rate. It was the consensus that polyspermy rate should be <6%. Observed values above this rate should be reported and investigated.

1PN rate following IVF or ICSI

The 1PN Rate Following IVF or ICSI PI (Table III) is defined as the proportion of inseminated oocytes with one pronucleus on Day 1 (17 ± 1 h post-insemination). It can provide a marker of a problem in gamete handling or culture conditions and so should be low under normal conditions. It was the consensus that the 1PN rate should be <5% for IVF cycles, and <3% for ICSI cycles. The difference between IVF and ICSI is related to the pre-selection of oocytes prior to ICSI injection. Observed values above this rate should be reported and investigated.

Failed fertilization rate (IVF cycles)

The failed fertilization rate (IVF cycles) KPI (Table IV) is defined as the proportion of IVF cycles (excludes ICSI cycles) with no evidence of fertilization (i.e. 0 oocytes with ≥2PN) on Day 1 (17 ± 1 h post-insemination). It can provide a marker of a problem in gamete quality (sperm function, oocyte activation, gamete receptors), sperm processing, or in the number of spermatozoa used for insemination. It should be low, under normal conditions. Based on the Alpha survey, and on the values recommended by the Association of Clinical Embryologists (Hughes and Association of Clinical Embryologists, 2012), it was the consensus that the IVF failed fertilization rate should be <5% for stimulated cycles. Observed values above this rate should be reported and investigated.

Zygote grade (IVF cycles)

Zygote grade is an evaluation of the quality of the fertilized oocyte, conducted 17 ± 1 h post-insemination. It was the consensus that there were not enough data to recommend indicator values for this measure. In the discussion related to micronuclei, it was agreed that while micronuclei are abnormal, there is no evidence to confirm the identity of these dynamic manifestations.

Early cleavage rate

Early cleavage rate is the proportion of fertilized oocytes that have undergone the first round of cleavage by 26 ± 1 h post-insemination by ICSI or 28 ± 1 h post-insemination by IVF. There is evidence that early cleavage, together with other factors, can be used as embryo selection method as it has been correlated with implantation rate (Shoukir *et al.*, 1997; Lundqvist *et al.*, 2001; Balaban and Urman, 2003; Ciray *et al.*, 2005; Brezinova *et al.*, 2009). However, as it is not routinely calculated, it was the consensus that while this indicator can be useful for troubleshooting purposes, there were no recommendations for expected values.

Cleavage rate

The cleavage rate KPI (Table IV) is defined as the proportion of zygotes that cleave to become embryos on Day 2 (44 ± 1 h post-insemination) (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011). It provides an indication of the ability of the culture system to support cleavage of fertilized oocytes (i.e. with occurrence of cellular division), and of embryo viability, so a low cleavage rate could be a warning that the culture system

has been impacted by an extrinsic factor. Furthermore, there is evidence that the presence of at least one non-cleaved embryo is predictive of reduced embryo quality for the remaining cohort (Machtinger *et al.*, 2015). The reference values are: competency > 95%; benchmark > 99%.

Embryo development rate

The KPI embryo development rate (Table IV) is defined as the proportion of cleaved embryos at the 4-cell stage on Day 2 (44 ± 1 h post-insemination) or at the 8-cell stage on Day 3 (68 ± 1 h post-insemination) per normally fertilized oocyte. This is an indicator of the ability of the culture system to support cleavage according to the expected stages, as well as providing an indication of the viability and quality of embryos. This KPI just considers the developmental stage of the embryo, regardless of grade, because developmental stage has been reported to give the highest degree of predictive power, and has the advantage of being an objective measure. It was acknowledged that not all clinics consider the specific cell stages defined here, and that the culture system used can affect the kinetics of embryo development.

The reference values, based on the median and maximum results from the participants' laboratories are: Day 2, competency ≥ 50% and benchmark ≥ 80%; and Day 3, competency ≥ 45% and benchmark ≥ 70%.

Notes:

– It was the decision of the expert panel not to consider Day 4 embryo development rate.
– The Embryo Development Rate KPI is most relevant to clinics that transfer embryos on Day 2 or 3.

Embryo and blastocyst utilization rates

These potential indicators were defined as the number of embryos (or blastocysts) suitable for transfer or cryopreservation as a function of the number of normally fertilized (2PN) oocytes observed on Day 1. Although it was agreed that these indicators could be of value for internal laboratory comparison, the consensus opinion was that because there are so many differences in laboratory and clinical practice, it was not practical to suggest any values for these indicators.

Blastocyst development rate

The KPI blastocyst development rate (Table IV) is defined as the proportion of blastocysts observed at 116 ± 2 h post-insemination as a function of the number of normally fertilized oocytes. It estimates the ability of the culture system to support blastocyst formation from fertilized oocytes (i.e. with formation of ICM, TE and a blastocoele cavity), and provides an indication of embryo viability. It should be noted that this definition only considers blastocyst formation, with no consideration of blastocyst-stage or blastocyst quality.

The reference values are: competency ≥ 40%; benchmark ≥ 60% (Day 5). A possible additional PI might be the development of an additional 10–15% blastocysts by 140 ± 2 h post-insemination (i.e. by Day 6).

Good blastocyst development rate

The good blastocyst development rate PI (Table III) is defined as the number of good quality blastocysts as a function of the number of normally fertilized oocytes. Blastocyst quality is as defined in the Istanbul consensus (Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011). This indicator estimates the ability of the culture system to support the formation of high-grade blastocysts from fertilized oocytes (i.e. with formation of ICM, TE and a blastocoele cavity), and an indication of embryo viability. The reference values are: competency ≥ 30%; benchmark ≥ 40% (Day 5). A possible additional PI might be the development of an additional 5–15% blastocysts by 140 ± 2 h post-insemination (i.e. by Day 6) (depending upon the culture system).

Proportion of good blastocysts

This PI is defined as the proportion of blastocysts with a grade of 'good' or higher. There was no discussion of reference values for this indicator, as they can be inferred from the preceding two Indicators.

Day 5 embryo transfer rate

This PI is defined as the proportion of cycles with at least one utilizable blastocyst on Day 5 relative to the presence of at least one 2PN oocyte on Day 1, to allow for the inclusion of cycles in which the decision has been made to cryopreserve all embryos. This indicator reflects the efficiency of the whole culture system, but is only relevant for those clinics that have a blanket strategy of Day 5 transfers. It was the consensus opinion that as there are too many clinic-specific variables, including different embryo transfer policies in different centers, clinics should develop their own expectations for this indicator, depending on when the decision to go to Day 5 transfer is made (e.g. Day 0 vs Day 3).

Implantation rate

For this consensus, the implantation rate KPI (Table IV) is defined as the number of gestational sacs divided by the total number of embryos transferred, irrespective of whether a pregnancy was established (Zegers-Hochschild *et al.*, 2009). There was some dissent about the use of sacs, rather than fetal hearts—which was felt to be a more meaningful indicator of implantation rate—however sacs are used by most national/international registries. Following discussion, it was agreed to use sacs for the purpose of this consensus, but that the use of fetal heart as the numerator should be revisited in the future.

Implantation rate provides an indication of the overall performance of the laboratory so an overall low implantation rate is a serious sign of a systemic problem. Values would be expected to be lower for Days 2 and 3 transfers than for blastocyst transfers. In addition, results will be influenced by clinical factors (e.g. uterine receptivity) and the existence of different policies in different centers for deciding the day of embryo transfer.

Reference values for implantation rates

Planned transfers of cleavage-stage embryos (Day 2 or 3): competency ≥ 25%, benchmark ≥ 35%.
Blastocyst transfers: competency ≥ 35%, benchmark ≥ 60% (the panel was divided between 55% and 60%, but agreed that 60% was an aspirational goal).

Note: These values could be affected if there are a large number of patients in the cohort who have had a large number of previous, unsuccessful cycles or significant clinical adverse factors. In addition, individual clinics may wish to further stratify their results based on patient age groups.

Live birth rate

It was the consensus that there are too many other variables to support the use of LBR as a laboratory indicator for either cleavage-stage or blastocyst-stage embryo transfers.

Successful biopsy rate

The KPI successful biopsy rate (Table IV) is defined as the proportion of biopsied and tubed/fixed samples where DNA is detected. It is a measure of the ability of embryologists to transfer the biopsied samples to test tubes, as proven by positive DNA amplification.

Based on data from the surveys and the PGD Consortium, which reported a 91% diagnosis rate in 254,820 biopsies (De Rycke *et al.*, 2015), the reference values were: competency ≥ 90%; benchmark ≥ 95% (Table IV).

No biopsy

This indicator was defined as the proportion of treatment cycles that had no embryos available for biopsy. It was the consensus that not having good quality blastocysts for biopsy is not an indication of the quality of the PGD/PGS service. It is an expression of blastocyst development rates, and so no further values were developed for this indicator.

No embryos found on warming/degenerated on warming

Nowadays, the majority of PGT cycles are based on blastocyst biopsy and cryopreservation. It was agreed that no embryos found on warming/degenerated on warming should be a KPI, as it provides a reflection of operator skills, and/or the device used. However, since not finding the embryo is a rare event, the panel was unable to estimate a competency value, as the value would be very low.

Owing to greater experience with blastocyst vitrification, the rate of degeneration on warming should now be lower than that estimated in the previous cryopreservation consensus (Alpha Scientists In Reproductive Medicine, 2012). Similarly, it was the consensus that re-expansion does not differ between (warmed) biopsied and non-biopsied blastocysts.

Notwithstanding some device differences, the reference rates for blastocyst cryosurvival could now reasonably be expected to be: competency ≥ 90%, benchmark ≥ 99% (Table IV).

Implantation rate of biopsied embryos

It was the consensus that the implantation rate for blastocysts biopsied for PGS should exceed that expected for the age-matched patient population in the same clinic. From the literature, a meta-analysis reported an improvement of 30% sustained implantation rate after the transfer of PGS-selected blastocysts relative to controls (Scott *et al.*, 2013; Dahdouh *et al.*, 2015).

Time-lapse imaging

Despite the increasing number of IVF cycles incorporating time-lapse imaging for embryo assessment and selection, the panel considered it premature to propose time-lapse related PIs for the IVF laboratory, owing to the limited and varied data associating precise timings of human embryo development with viability or good laboratory practice. It was accepted, however, that because of the detailed morphological and kinetic information collected per embryo, time-lapse imaging may prove to be a future early warning tool for compromised culture conditions, providing a (intra-)laboratory PI, specifically if a change in mean timings for embryos to reach developmental milestones may be detected more readily and rapidly than with standard assessment methods.

A time-lapse assay of mouse embryo development linked specific morphokinetic changes to toxicity of mineral oil. This demonstrates the sensitivity of mouse embryo cleavage timings to the quality of the culture environment, and the potential value of time-lapse in detecting such changes (Wolff *et al.*, 2013). To date, no studies of this type have been performed on human embryos.

The current recommendation, therefore, was that clinics may wish to establish time-lapse KPIs and benchmarks based on their own experience. It was suggested that the frequency of anomalous cleavage events, such as trichotomous mitosis (direct cleavage to three cells), which is known to be relatively common (6–8%) and associated with reduced implantation potential, could be monitored for future use as a KPI (Rubio *et al.*, 2012; Athayde Wirka *et al.*, 2014). In addition, it was considered that clinics using validated time-lapse algorithms for embryo selection may develop benchmarks associated with the proportion of embryos which ranked highest using morphokinetic modeling. Large-scale population studies are, however, required in order to identify which, if any, morphokinetic markers can be developed into universal and useful laboratory KPIs. It is also important to point out that morphokinetic embryo performance is believed to be highly associated with clinical and laboratory practices, and may, therefore, be difficult to compare between laboratories.

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2

Clinical Aspects of Time Lapse Imaging

Alison Campbell

Introduction

Morphological assessment of the preimplantation human embryo is the single and most commonly used method employed for embryo selection following IVF, and the correlation between embryo morphology and implantation potential has been extensively demonstrated and documented.

Typically, this morphological evaluation consists of up to six single, daily, conveniently scheduled observations during in vitro development to the blastocyst stage. Although, as stated, selection of the embryo(s) for transfer tends to be weighted toward the morphology of the embryo just prior to embryo transfer, other factors play a significant role. Assessing cell number at a particular time of development and the amount of fragmentation have led to simple embryo grading schemes, such as that utilized within the United Kingdom.[1] Other schemes include a more graduated approach to embryo scoring that takes into account several other variables such as pronuclear morphology.[2] More recently, the Istanbul consensus workshop on embryo assessment aimed to define common terminology and the minimum criteria for oocyte and embryo morphology assessment to allow effective comparisons and standardized reporting.

This published evidence-based grading scheme relies on morphological parameters as well as developmental stages being reached, each of which have been proven to be associated with embryo viability.[3,4]

Failure to select a viable embryo from a cohort will inevitably limit the chance of a positive outcome. The unwitting selection of a nonviable embryo, and the cryopreservation of viable alternative(s) within a cohort, will also delay the desired outcome. There is rapidly growing evidence to support that more emphasis should be given to the selection of embryos displaying optimal morphokinetic characteristics; however, assessment of morphokinetics (the morphology and dynamic behavior of the embryo, studied over time) is not possible when using standard incubation accompanied by static microscopical methods. The limited observations employed to minimize environmental stress to the embryos using this traditional 'snapshot' approach provide comparatively limited information on developmental rates or patterns.

The introduction of time-lapse microscopy as a clinical tool in IVF has resulted in several reports of improved embryo selection based on the analysis of sequential time-lapse images of embryo development. Time-lapse devices, now commercially available for the IVF laboratory, allow the capture of images of the embryos developing in vitro at regular intervals throughout the culture period. This provides continuous and uninterrupted monitoring that furnishes embryologists with a more sophisticated and promising tool for the study and selection of the human preimplantation embryo beyond conventional daily microscopy. The EmbryoScope was the first instrument to provide stable, uninterrupted incubation combined with internal microscopy. Meseguer et al., using this system, observed a 20 per cent increase in pregnancy rate compared to standard incubation and attributed this improvement to the EmbryoScope's stable culture conditions and use of morphokinetic variables for embryo selection.[5] Time-lapse technology also allows the identification of aberrant embryo cleavage that would not be observable using static traditional methods. An example of aberrant cleavage, which is reportedly common, is the phenomenon of a direct, or rapid, cleavage to three cells—in less than 5 hours.[6] This study demonstrated the ability of time lapse to identify aberrant cleavage divisions and highlights the reduced implantation potential when such embryos were transferred compared with embryos that did not exhibit this behavior. In a cohort of 1,659 transferred embryos, the incidence of this 'direct division' was 13.7 percent, and the known implantation rate of these embryos was markedly and statistically significantly lower than for embryos with a normal cleavage pattern (1.2 versus 20.2 percent, respectively). Importantly, this aberrant, but relatively common and clinically significant, cleavage had not been identifiable prior to time lapse being available.

There are multiple applications for time-lapse monitoring within the IVF laboratory. These include validation of conventional static assessment methodologies, prediction of embryo viability and outcome, comparison of the potential impact of variables such as culture media or drug regimens on morphokinetics, observation of transient or aberrant morphological phenomena, quality control, and improvement of flexibility and working patterns. This chapter considers practical aspects of time-lapse imaging, considers the use of morphokinetic data to develop embryo selection algorithms, and discusses how time lapse can be incorporated into the IVF laboratory in order to increase flexibility, information, and outcome for IVF patients.

Selection and Deselection Morphokinetic Criteria

Time-lapse monitoring of the dynamics of embryo development, in addition to traditional qualitative morphological observations, provides morphokinetic information on individual embryos. These data, generated by the manual or automatic recording (annotation) of the images, collected over precise time points, can be retrospectively analyzed against outcome variables, such as blastulation implantation, ploidy, or live birth. This type of analysis may allow practitioners to identify prospective and preferential selection criteria for embryo transfer or cryopreservation.

It is also effective in identifying exclusion, or 'deselection' criteria. For example, if a morphokinetic variable or event is shown to be associated with very poor clinical outcome, or even preclude implantation, embryos exhibiting this can be deselected for transfer, irrespective of their traditional morphological score.

Data Handling

Morphokinetic data from time-lapse monitoring systems should be precisely grouped according to known outcome following embryo transfer, for comparison and statistical analysis. Specific attention is required to exclude data following multiple embryo transfer that resulted in a lower number of fetal hearts or babies born than the number of embryos transferred. Additionally, inclusion of double embryo transfer morphokinetic data where two implantations resulted may be problematic without the use of genetic fingerprinting to ascertain the chorionicity or zygoticity of the pregnancies. Due to the ranges observed for each morphokinetic variable, it is recommended that median values be used for comparison between successful and unsuccessful groups of embryos as opposed to means in order to avoid skewing the results. Once key significant variables have been identified, depending on the power of the data set available, they can be ranked according to their association with outcome and simple embryo selection algorithms, or models, developed.

Embryo Selection Algorithms

IVF clinics already use evidence-based biomarkers of embryo quality to aid their decision as to which embryo should be transferred or cryopreserved (e.g., early cleavage to two cells, presence of smooth endoplasmic reticulum aggregates), but this practice has limitations based on the static nature of the observation and often transient nature of the markers. Nevertheless, the embryologist may elect to use these existing selection or deselection criteria while building experience and data with a newly introduced time-lapse system, and while their individual, specific criterion is fine-tuned over time. For example, rather than using the current consensus value of 25 ± 1 hour, postinsemination, for early cleavage observation on day 1, as defined by the embryo grading and assessment guidelines, a more precise time point for this event, perhaps incorporating the insemination method, or patient factors, will in time be established for that setting which relates to outcome.[3,4] It may then be incorporated into an embryo selection model.

The first morphokinetic-based model, which used specific timings to predict embryos most likely to develop to the blastocyst stage, was reported by Wong and colleagues in 2010.[7] Since then, prediction of blastulation, as a measure of embryo quality, or viability, has now been superseded by more robust clinical outcome measures such as ploidy, implantation, and live birth.[8-14]

Meseguer and colleagues used a hierarchical approach to modeling such that embryos received a classification based primarily on developmental milestones and relative timings associated with them. In a retrospective analysis of over 500 transferred embryos, where specific implantation data were available for 247, they found significant differences between implanted and not implanted embryos for six early morphokinetic variables, the most significant for implantation prediction being the time the embryo reached the five-cell stage (t5) and the duration of the second cell cycle (cc2). Their published model included three exclusion criteria based on their negative association with implantation. These were direct cleavage from one to three blastomeres, uneven blastomeres, and multinucleation at the four-cell stage.[8] More recently, Campbell and colleagues considered whether there was a difference in the morphokinetics of euploid and aneuploid embryos using data from blastocysts that had undergone biopsy and preimplantation genetic screening (PGS).[9] The only significant variables identified, from more than 20 variables compared, were the time to start blastulation (tSB) and the time to reach the full blastocyst stage (tB) defined according to their annotation system. From this, using recursive partitioning, they presented a risk classification model for aneuploidy.[9] More recently, Basile and coworkers have proposed a logistic regression–based model to improve the chance of selecting a euploid embryo from a cohort, based on morphokinetic data from embryos that underwent blastomere biopsy and PGS. They reported that chromosomally normal and abnormal embryos have different kinetic behavior and based the model on the significant morphokinetic variables t5-t2 and cell cycle three (cc3 = t5-t3).[15] Even though euploidy is crucial for optimum live birth outcome, there are clearly many more factors required within the embryo itself, and maternally, in order to ensure viability. For this reason, healthy live birth, rather than ploidy, is the ultimate outcome measure. Live birth was used as an outcome measure in a multiplicative model for early cleavage embryo selection. In this model, key variables and timings or intervals were weighted in order to create a ranking of embryos based on their predicted live birth potential. When morphokinetic profiles were compared between embryos that resulted in live birth and those that did not, the significant morphokinetic variables identified related to the first cleavage and early cell cycle lengths. Although a small study, this model is promising and was demonstrated to be very predictive of live birth as measured by the area under the receiver operating characteristic (ROC) curve (value was 0.8).[14]

Despite a number of morphokinetic embryo selection models now being published, it should be noted that such models may not be directly transferrable to another clinical setting. Inability of a model to be effectively reproduced in an alternative setting

may be due to confounding factors, such as culture environment. For example, partial pressures of incubation gases or media used have been reported to influence morphokinetics, as have patient factors such as age and body mass.[16-18] It was recently demonstrated that the multivariate hierarchical selection model described above was not transferable from one clinical setting to another without modifications.[19] Conversely, the aneuploidy risk classification model, also referred to above, was effectively tested on large and independent data in relation to implantation outcome.[20] Whether pre-embryonic genome activation events, such as cleavage morphokinetics prior to the eight-cell stage, or later morphokinetic information representing the activated embryonic genome will give the most reliable selection criteria needs further study. Most published work to date has focused on events up to the five-cell stage, and as a prognosticator for blastulation, or implantation, rather than live birth.[7,8] While it is recognized that maternal effects may mitigate against the survival of the viable embryo, in any event—and not least because aneuploidy is the largest single cause of failure—live birth outcome, in relation to morphokinetics as a selection tool, should be the gold standard measure. While arguably easier, and consequently more objectively interpreted for annotation, they may not be as reliably representative of onward embryo development and potential following activation of the embryonic genome.

Need for Consensus

Consensus guidelines for professionals working with time-lapse monitoring in IVF do not exist currently, although with training workshops and forums often facilitated by device suppliers, there are opportunities for embryologists and researchers to compare and debate best practice. With an inevitably increasing proportion of IVF cycles now being conducted using time-lapse imaging, the need for professional guidelines for definition, interpretation, and annotation of images becomes a priority. Annotations are the records of morphological and dynamic events that provide the basis on which individual embryos can be evaluated and selected using defined criteria. Consistency in annotation, within the clinic, and the field will allow alignment of effective morphokinetic embryo evaluation.

The process of reaching proposed consensus for morphokinetic embryo selection, and acceptance of it, will be challenging due to the already existent heterogeneity within the published time-lapse studies and the lack of prospective randomized controlled trials to fully support the concepts. Many clinics experience difficulty in recruiting patients into these studies; this classical measure of 'evidence-based' data may delay consensus for morphokinetic embryo selection. However, these would be facilitated hugely by consensus guidelines for the annotation of time-lapse images and the definitions used to describe morphokinetic variables.

Second Opinions and Training

One of the key advantages of time-lapse systems in the IVF laboratory is the facility to allow clinical embryologists to rewind, pause, and review photographic frames in order to consider the detail and context of embryo development with practical flexibility without interruption of embryo culture. The storage of images also allows them to be reviewed retrospectively in order to reannotate further detail and perhaps consider novel events that went unrecorded previously (as noted in this atlas). It may be particularly worthwhile to annotate additional variables, for example, for embryos with known outcomes only. This would provide clinical relevance and possibility in the search for morphokinetic biomarkers for live birth outcome.

Quality Control and Assurance

As with many aspects of the embryologist's role, there are practical and efficiency benefits if multiple individuals are trained and competent in routine daily tasks. Time-lapse annotation is no exception, and once introduced into the laboratory, review, annotation, and interpretation of time-lapse images is currently considered a daily task. Where there are multiple practitioners involved, the risk of subjectivity and inconsistency is highest, although intrapractitioner annotation variation may also exist. Ensuring the most accurate and objective record of dynamic, often anomalous, embryo development brings challenges whether using automatic detection software or solo or collective manual methods. In order to minimize subjectivity, it is recommended that key variables for annotation be defined within the standard operating procedure (SOP) and that these be routinely recorded. With the current lack of consensus, policy should be set in house, adhered to, monitored, and refined where required. Core morphokinetic variables should be identified and annotated following rigorous training and competency assessment. Some morphokinetic variables are more at risk of subjective interpretation than others (e.g., the appearance of pronuclei). The use of reference images may be beneficial in assuring annotation quality.

The most commonly recorded morphokinetic variables follow the basic principles of embryology and mitosis and include timing of pronuclear appearance and fading, increasing cell numbers (time to two, three, four, five, six cells, etc.), and times of embryo differentiation to the morula and blastocyst stages. Durations of mitotic cycles and synchronicity can then be calculated from these. In addition, specific anomalies or phenomena associated can be annotated, depending on the customizability of the time-lapse system available.

Once established, a regular audit of annotation completion and quality and adherence to SOP is essential in order to maintain high-quality data to allow analysis and opportunity for identification of significant selection or deselection morphokinetic criteria.

Early indications are that many of the morphokinetic events are recorded objectively, but it is critical that regular review and assessment exercises should be undertaken to assure quality. A study by Sundvall and colleagues considered inter- and intrapractitioner variation in annotation, using the intraclass correlation coefficient.[21] Their study demonstrated a close correlation between both experienced and new time-lapse users for most morphokinetic variables but highlighted that some 'static morphologic parameters' such as multinucleation and blastomere

evenness remained at risk of subjectivity. With ongoing assessment and clear definitions and SOP, this can be minimized. Another report also found close correlation between annotators but highlighted how one misannotation could skew the data output.[22] In summary, until we know the potential impact of subtle deviations from protocol, culture dishes or slides should be prepared in a standard and precise fashion, and annotation of time-lapse images, where performed manually, performed with objectivity by all practitioners.

Flexibility and Opportunity

Due to the great interest in time-lapse technology by professionals in the field of IVF, the promising clinical results, opportunities to broaden knowledge of the preimplantation embryo's development, and its visual nature which appeals to clinic staff and patients, swift advances in the technology and the application of it are inevitable.

Computer servers, rather than stand-alone devices, will allow the support of new applications such as patient interface, improved data collection, storage, sharing, and remote access.

The Zoi server (FertiliTech, Denmark) is such a platform that supports secure data sharing within and between clinics using EmbryoScope. Patient data can be collected from multiple connected incubators and centralized in a common storage location. This enables IVF practitioners to view, annotate, and select embryos remotely which opens up new opportunities for productivity and flexibility. Before too long, time-lapse users may also expect statistical software packages for quality assurance and detailed analysis of data with associated embryo selection model building.

Summary

Within this rapidly progressing and promising area of reproductive medicine, practitioners now have an additional and increasingly reliable tool for improving embryo selection. It is yet unclear as to which patients may benefit most from this new approach, and reported significant uplifts in pregnancy and live birth rates by the use of this technology are still to be proven in large, randomized, controlled trials. However, it makes sense to observe and interpret the dynamic process of preimplantation embryo development, using technology that allows the dynamics to be studied, rather than a rigid and static, snapshot method, not least for the anomalies that can be excluded. The more we interrogate the collated images and data output, the more we can understand whether an optimal morphokinetic profile exists. The technology has the potential to provide embryo selection algorithms such that numerous criteria will be defined for a range of varying circumstances, from individual patient criteria to generalized laboratory conditions. In time, the optimal ranges for defined dynamic events such as those directly associated with the 'normal' cell cycle may be fine-tuned and further novel morphokinetic markers of embryo viability identified.

Time-lapse monitoring is a tool that not only dramatically increases flexibility in the IVF clinic but also has the potential to train, educate, and most importantly enhance clinical outcome.

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3

Time Lapse, the Cell Cycle, Distribution of Morphokinetic Timings, and Known Implantation Data

Alison Campbell

Cell Cycles and Time Lapse Monitoring

A cell cycle is a series of complex events involving cellular and nuclear processes through particular phases that ensure the cell's division into two daughter cells. Mitosis consists of nuclear division and cytokinesis and consists of several phases: prophase, prometaphase, metaphase, anaphase, and telophase. These are preceded by interphase which encompasses stages GAP-1 (G1), Synthesis (S), and GAP-2 (G2) of what is known as the cell cycle. The duration of cell cycles in the human preimplantation embryo appears to be related to embryo viability.[1] Prolonged or rapid cell cycles could be associated with DNA repair, cellular rearrangements, or failure of an embryo to undergo cell cycle checkpoints. Both cycles could potentially compromise embryo development.[2]

The first cell cycle following fertilization has recently been described in detail following a large time-lapse study by Aguilar and colleagues.[3] In their study, they described initiation of the first cell cycle as the time from the completion of the second polar-body extrusion. The length of the S-phase (DNA replication) was defined as the time from appearance to the fading of two pronuclei. In this study, embryos with prolonged S-phase demonstrated significantly reduced implantation rates compared with embryos with S-phases ranging from 5.7 to 13.8 hours. Completion of the first cell cycle can be defined as the time point at which the embryo reaches the two-cell stage, such that the two daughter cells are discrete from each other.

Postinterphase cleavages of corresponding sister blastomeres rarely occur at precisely the same time, and so time-lapse users may also consider the synchronization of cell divisions within a round of mitosis, particularly as this has been reported to be correlated to embryo viability. The optimal value for this being reported is less than 0.76 hours.[4] This can be calculated by the synchrony of sister cell divisions. For example, synchrony of the second cell cycle would be defined as the duration of the transition of an embryo from two cells to four cells and is calculated by subtracting the time the embryo reached the three-cell stage (t3) from the time it reached the four-cell stage (t4). With frequent time-lapse image acquisition (such as every minute), the duration of each cytokinesis can also be measured from the time a cleavage furrow is first visible to the time that the daughter blastomeres are discrete from each other.[5]

Cell cycle duration is calculated using time-lapse annotation either according to a single cell division or as a round of mitosis whereby the number of blastomeres doubles. For the first cell cycle, as development begins with the single cell, these are the same. However, the second cell cycle begins with two cells, both of which should subsequently divide, forming two daughter cells each. There are therefore two individual blastomere cell cycles but a single embryo cell cycle, and this results in the doubling from two to four cells.

Figure 3.1 provides a schematic to represent the blastomere cell cycles (cc) and the rounds of divisions herein defined as embryo cell cycles (ECCs), resulting in the doubling from two to four and from four to eight cells. The cell cycle for blastomere *a*, is calculated as t3-t2 and documented as cc2a, and for blastomere *b* as t4-t2 and documented as cc2b. The cell cycle whereby the embryo reaches four cells from two cells (ECC2) is also calculated as t4-t2. The time that the last cleaving blastomere takes to cleave (from t2 to t4) equates to the duration of the ECC: All individual blastomeres cleave within this time frame. The same applies for the third cell cycle. The duration of the embryo's third cycle (ECC3) is the time it takes the embryo to develop from four to eight cells and includes four blastomere/cell cycles: *a*, *b*, *c*, and *d*. The cycle cc3a is t5-t4, cc3b is t6-t4, cc3c is t7-t4, and cc3d is t8-t4. Cycle ECC3 is t8-t4. The synchronization for ECC3 (S3) is calculated as t8-t5 as the period begins once the first of the four cells cleaves (Figure 3.1). Video 3.1 shows an oocyte post ICSI as it undergoes fertilization and cell division to a hatching blastocyst. The video pauses at the described morphokinetic time points; t0 is normally considered as the time at insemination (ICSI). The video can be viewed online: http://goo.gl/U6TN1n.

Morphokinetic markers depicted in Figure 3.1 and Video 3.1 are defined as follows, and unless otherwise stated, are considered as time post insemination (t0), generally recorded in hours; time to pronuclear fading (tPNf); time to two cells (t2), three cells (t3), four cells (t4), five cells (t5), and so forth; morula (tM); start of blastulation (tSB); and full blastocyst (tB).

Known Implantation Data (KID)

The precise timings or durations of an embryo's development, its pattern and movement (kinetics), alongside its morphology have

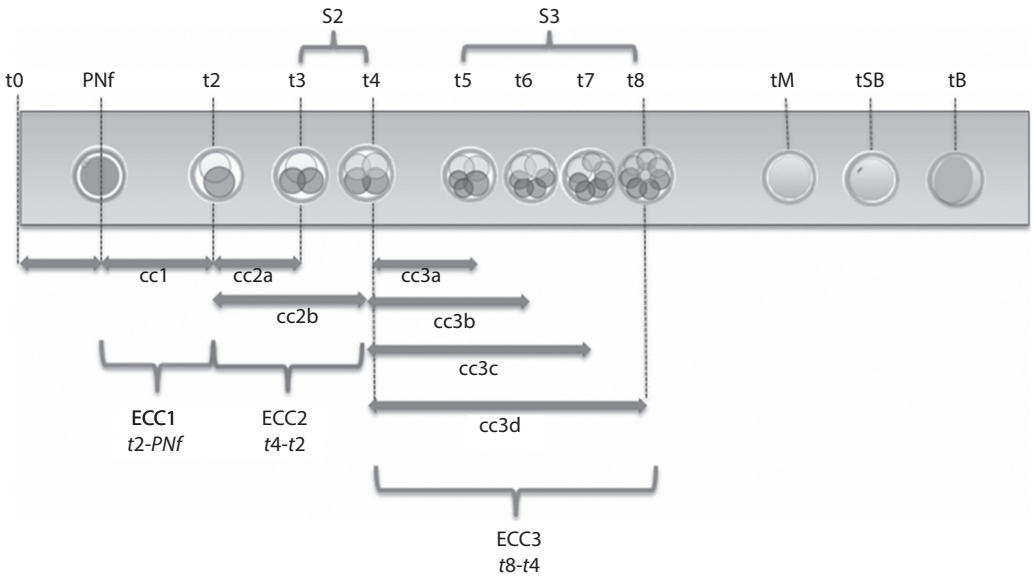

Figure 3.1 Three embryo cell cycles (ECC1, ECC2, and ECC3).

been referred to as its *morphokinetics*.[6] The morphokinetic data of a specific and transferred embryo which has a known outcome is commonly referred to as *known implantation data* (KID). The known outcome may be a pregnancy test result (positive or negative), a gestational sac or fetal heart on ultrasound scan, a pregnancy loss, or a live birth. Morphokinetic data can be compared between embryos giving positive or negative implantation data (KID+ or KID−), depending on the outcome measure used. All data can be utilized following a single embryo transfer, or a double embryo transfer with a negative outcome as the fate of these embryos is known. Using data, following multiple embryo transfer that has resulted in the same number of fetal hearts or babies born, may be problematic without the use of genetic fingerprinting to ascertain the chorionicity or zygoticity. However, in time, the level of monozygotic twinning can be established and factored to assess its significance and thereby the need for DNA fingerprinting.

Due to the ranges observed for each morphokinetic variable, it is recommended that median values are used as opposed to means. This way, extreme high or low values outliers do not introduce a skew.

The KID positive rates (or ratios) can be calculated for each morphokinetic variable in order to consider their impact and potential use in embryo selection models. Varied mathematical approaches, including recursive partitioning, have then been used in model development in order to ascertain significant and optimal time values, or ranges, for building embryo selection algorithms.[7]

Figure 3.2 shows a schematic representation of KID.

Distribution of Timings of Cleavages

These data represent over 9000 embryos (in vitro fertilization [IVF] and intracytoplasmic sperm injection [ICSI]) that underwent time-lapse monitoring at the CARE Fertility Group, United Kingdom, using EmbryoScope (FertiliTech, Denmark) technology. The blue bars represent the numbers and morphokinetic timings for each variable of embryos which implanted, defined by the presence of a fetal heart on ultrasound scan (7 ± 1 week gestation). Timings are given in hours post insemination (hpi). Embryo culture was performed in microwells of 25 μL Global IVF medium (LifeGlobal®) supplemented with 10 percent Dextran serum supplement (Irvine Scientific), overlaid with 1.4 mL mineral oil (Fertipro, Belgium). Embryo culture took place at 37°C in 5.5 percent CO_2, 5 percent O_2, and 89.5 percent N_2. It is important to note that ranges of morphokinetic variables may vary according to gas tension, culture media, or patient factors, and clinics should consider their own data to establish expected ranges within their clinical practices.

The morphokinetic variables used in this evaluation are described within the text and in Figure 3.3. Durations of a distinct cell stage (or series of cell stages) can be obtained by subtracting one cleavage time from a later one (e.g., t7-t6 = duration of the six-cell stage).

Figure 3.3 shows a schematic representation of commonly used morphokinetic variables and calculated durations.

Time to Two Cells (t2)

The distribution of timings for the morphokinetic variable t2 in all embryos and in KID-positive embryos is shown in Figure 3.4.

Figure 3.4 supports the consensus based on static methodology that late t2 is associated with reduced implantation potential.[8] The upper limit for t2 in KID-positive embryos is 31 hpi; however, the majority of the embryos here were not transferred. Figure 3.4 also shows that first cleavage prior to 21 hpi is relatively uncommon at 1.2 percent of t2 data (n = 9,391) and that embryos that undergo this first cleavage prior to 21 hpi may have a lower incidence of known implantation compared to embryos with t2 in the 21 to 31 hpi range.

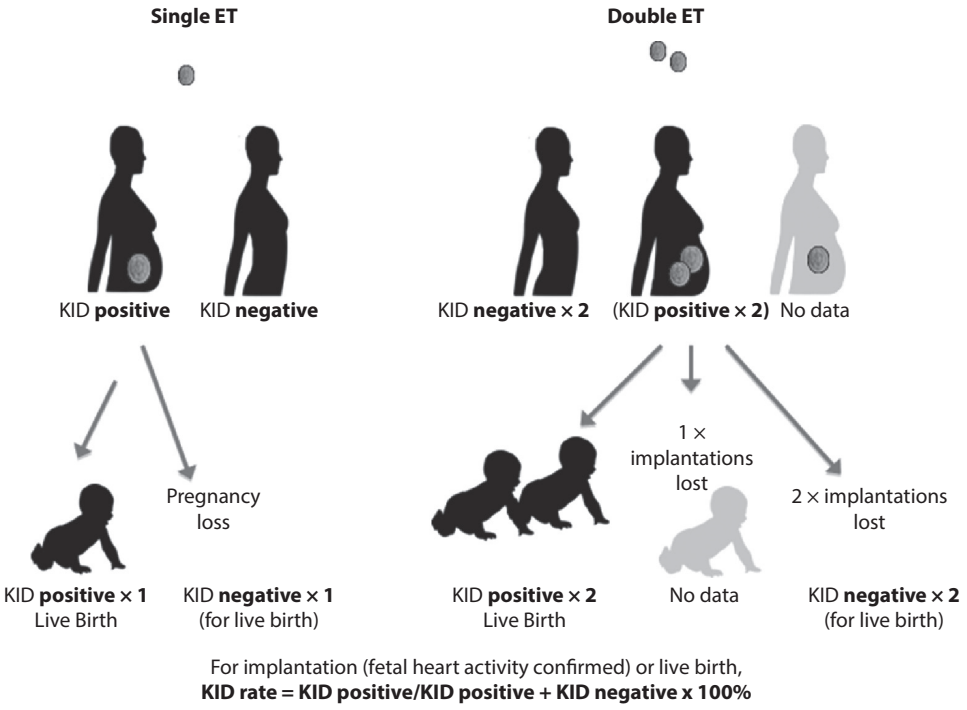

Figure 3.2 Schematic KID. (Modified from Alison Campbell, Non-invasive techniques: embryo selection by time-lapse imaging. In: Montag M, ed. *A Practical Guide to Selecting Gametes and Embryos.* Boca Raton, FL: CRC Press; 2014.)

Figure 3.4 shows a distinct range of t2 for preferential embryo selection and demonstrates how dynamic assessment of t2, as an example, provides practitioners with a tool that may allow the deselection, or exclusion, of embryos with t2 outside of a range that correlates to increased known implantation rates.

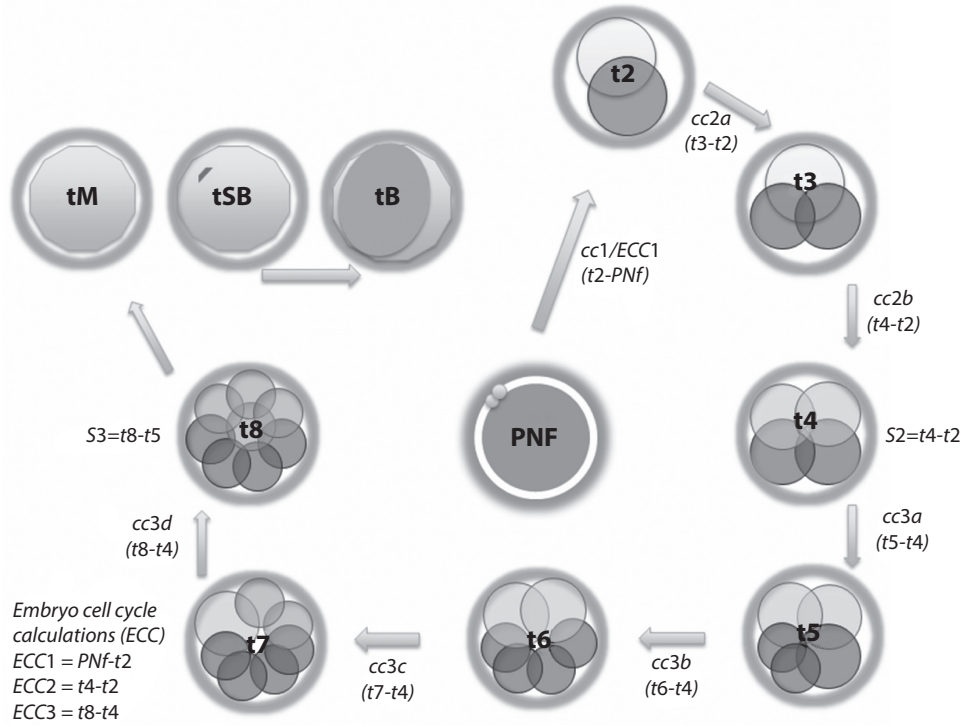

Figure 3.3 Morphokinetic variables.

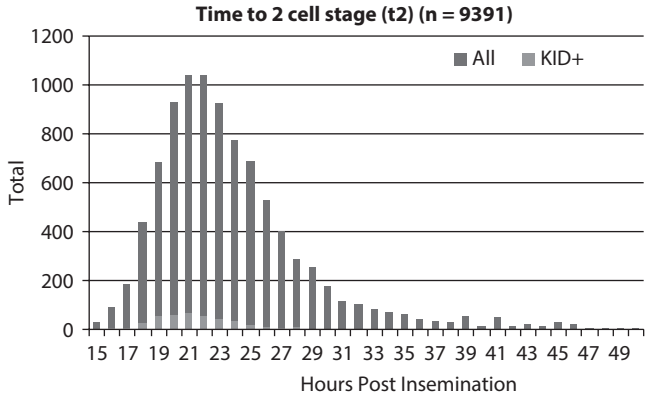

Figure 3.4 Distribution of timings for the morphokinetic variable t2 in all embryos and in KID-positive embryos.

Time to Three (t3) and Four (t4) Cells

The non-Gaussian distributions of timings for the morphokinetic variables t3 and t4 are shown in Figure 3.5. Optimal ranges for t3 and t4 are observed where the highest proportions of KID-positive embryos are present. These correspond, however, to the mode value for embryos overall.

Time to Five (t5) and Eight (t8) Cells

The non-Gaussian distribution of timings for the morphokinetic variables t5 and t8 are shown in Figure 3.6. The ranges for KID-positive embryos at t5 and t8 are broad, and the pattern of distribution, compared to embryos overall differs from those observed in Figure 3.5 for t3 and t4.

Time to Start Blastulation (tSB) and Time to Full Blastocyst Stage (tB)

The non-Gaussian distributions of timings for the morphokinetic variables tSB and tB are shown in Figure 3.7. For these variables, in contrast to the chronologically earlier variables discussed, the median values for tSB and tB in the KID-positive cohorts are earlier than the median values for the overall cohorts. These two morphokinetic variables were demonstrated to be delayed in preimplantation genetic screening (PGS)–confirmed aneuploid embryos compared with their euploid counterparts and have therefore been identified as potential morphokinetic variables for blastocyst selection models.[7]

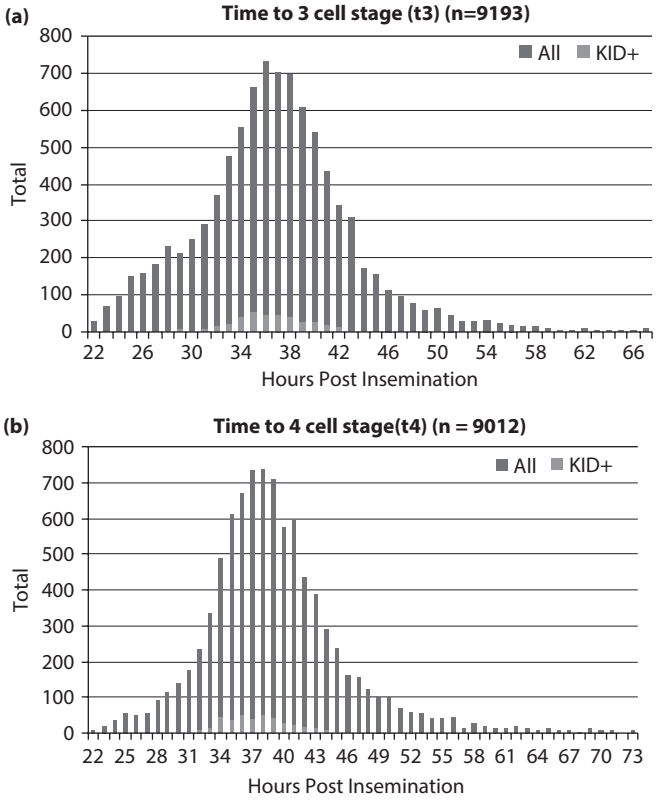

Figure 3.5 (a) Distribution of timings for the morphokinetic variable t3. (b) Distribution of timings for the morphokinetic variable t4.

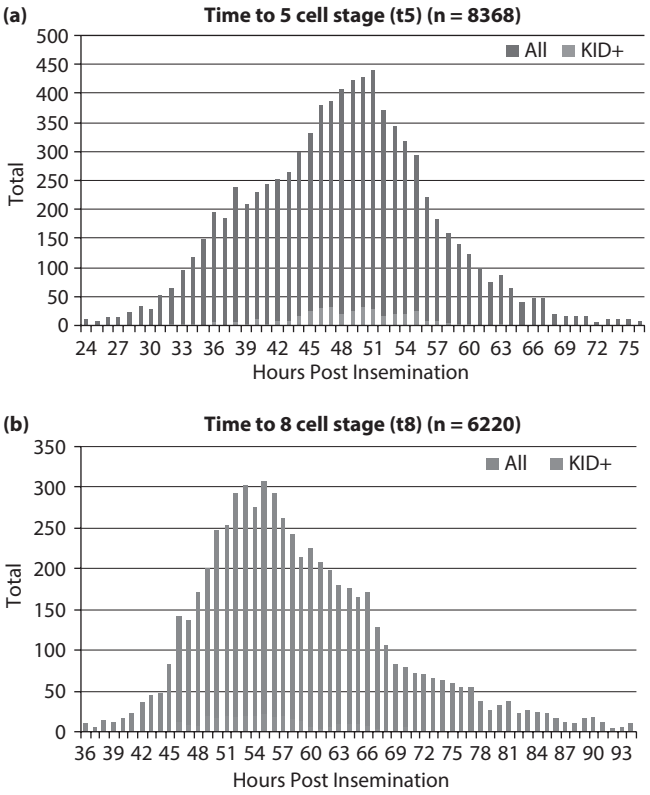

Figure 3.6 (a) Distribution of timings for the morphokinetic variable t5. (b) Distribution of timings for the morphokinetic variable t8.

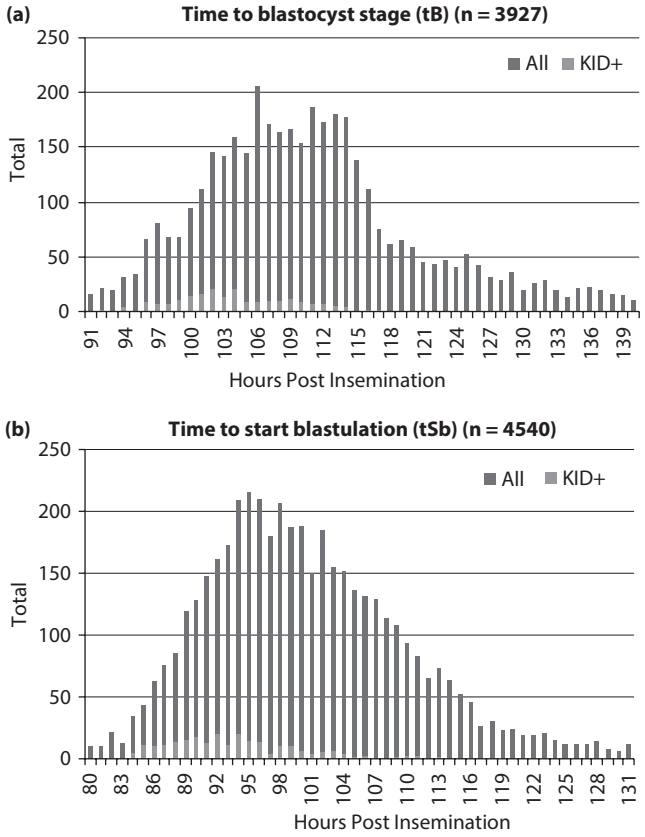

Figure 3.7 (a) Distribution of timings for the morphokinetic variable tSB. (b) Distribution of timings for the morphokinetic variable tB.

Second Cell Cycle (cc2)

Time-lapse studies of rapid cleavage events to the three-cells stage (in less than 5 hours) have demonstrated this to be a morphokinetic criterion significantly associated with reduced implantation rates. Embryos displaying this irregular division pattern, described as *direct cleavage*, gave rise to implantation rates of 1.2 percent compared with 20.2 percent for embryos not displaying this division pattern.[8]

Analyses of the duration of the second cell cycle, in over 9000 embryos, at the CARE Fertility Group show a bimodal distribution with differing variance; the direct division from one to three cells (trichotomous mitosis), associated with the first peak, was less frequent than the division pattern when two cells were reached as an intermediate stage (Figure 3.8). The irregular division pattern in this cohort, represented by the first peak, is also associated with significantly reduced implantation.

This large analysis demonstrates that 26.3 percent of embryos undergo irregular cleavage, either directly (or rapidly) from one to three cells in less than 5 hours. Such events are not observable using static conventional assessment.

A peak for optimal cc2 range is observed where the highest number of KID-positive embryos are present (Figure 3.8).

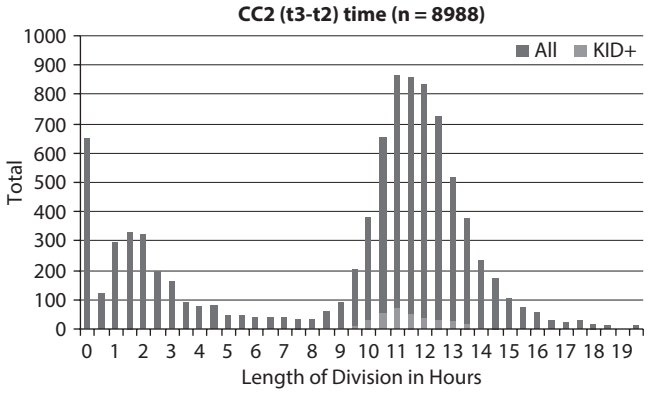

Figure 3.8 Second cell cycle (t3-t2) time (*n* = 8,988).

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Chapter 10
Prediction of Embryo Viability by Morphokinetic Evaluation to Facilitate Single Transfer

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Introduction

Optimal culture conditions and reliable embryo selection constitute two major challenges for successful IVF treatment. Embryo quality is typically assessed by the use of grading systems based on morphological evaluation under a microscope at certain, distinct time points. This methodology has several limitations. The inability to accurately assess embryo quality constitutes a hindrance for evaluating the impact of culture conditions and for estimating the reproductive potential of an embryo. The recent development of clinical time-lapse instruments has enabled continuous monitoring of human embryos, hereafter referred to as time-lapse imaging (TLI). TLI, where consecutive images are obtained during embryo culture by using a microscope and a camera, allows for a refi ned evaluation of known morphological parameters and represents a new method of evaluating embryo viability. Several retrospective studies have demonstrated a correlation between timing of key events and developmental or implantation potential, which suggests time-lapse imaging as a promising method for a more reliable embryo selection than morphology alone. However, as we expand our knowledge of pre-implantation embryo development, it becomes increasingly clear that timing is infl uenced by several patient- and treatment- related factors. This may complicate the establishment of a prediction

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model for optimal embryo development that may be applied under a variety of conditions across heterogeneous patient groups. This chapter addresses the use of TLI in the evaluation of pre-implantation embryo development and pregnancy potential in an effort to provide an overview of the feasibility and potential use of TLI in IVF treatment.

Scoring of Static vs. Dynamic Parameters

Traditionally, the quality and viability of pre-implantation embryos are evaluated by a microscopic inspection at a few, well-defi ned discrete time points. There is a welldocumented close correlation between morphological appearance and developmental stage of the embryo at given time points and developmental competence (as reviewed by ALPHA and ESHRE [1]). Due to the simplicity and cost-effectiveness of static morphological grading and lack of documentation for existing alternative methods, traditional morphological evaluation therefore remains the choice method for embryo evaluation. Nevertheless, this approach has several recognized limitations. Firstly, the information obtained with a few, discrete time point provides an incomplete picture of the inherent dynamic process of embryo development, as illustrated by the observation that embryo score may change markedly within a few hours [2]. This limitation is obviously overcome with continuous monitoring. Furthermore, morphological scoring of embryos has shown substantial inter- as well as intra-observer variation, which in turn has implications for the decision to transfer, cryopreserve, or discard the embryos [3 – 5]. A probable cause for this variation is that assessment in categories

tends to be rather imprecise. In contrast, the assessment of time-lapse parameters appears to have a high degree of intra- and interobserver agreement [6]. Theoretically, this agreement will depend on the instrument used, in particular the resolution, the number of focal planes, and the intervals between the photographic recordings. Any variation in clinical decision-making remains to be assessed, as no model has presently been prospectively validated, as discussed in detail below. TLI necessitates periodical light exposure, use of moving devices, and magnetic fi elds that constitute potential risks to the embryos. The safety of TLI for IVF has been documented in two trials conducted with the same instrument. Embryo development was the primary endpoint in both trials [7 , 8]. As for any new method introduced in the ART laboratory, a suffi ciently powered study using pregnancy rate or live birth rate with pediatric follow-up would be preferable before any defi nitive conclusions are drawn. Likewise, it must be noted that both trials were conducted using the same TLI instrument and that the conclusions may not necessarily extend to include other systems.

Introduction to Time-Lapse Parameters

While time-lapse monitoring is a rather novel method in the ART lab, the method has been used for nearly a century to study embryo development for research purposes [9]. Prior to the introduction of clinical instruments, research was conducted

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on embryos from various animal species or more seldom, surplus human embryos. Initially, the studies were aimed at describing the process of development, but as IVF was introduced, the attention was directed toward the potential use of time- lapse imaging to characterize division patterns and dynamic parameters that potentially would identify embryos that are viable beyond the time of observation. The following section describes typical in vitro development of a pre-implantation human embryo and the events that are visible and thus recordable in a time-lapse analysis.

Development of a human embryo begins with fertilization. The spermatozoon penetrates the extracellular multilayer glycoprotein coat, zona pellucida (ZP) [10], and the spermatozoon membrane fuses with the oocyte membrane [10]. The associated formation of the male pronucleus can be visualized with time-lapse monitoring. During normal fertilization the fusion of the two membranes initiates oocyte activation, leading to the completion of the second meiotic division of the oocyte. This stage is visualized by the extrusion of the second polar body 3–7 h after fertilization [11] followed by the visible formation of the male and female pronuclei. The male and female pronuclei (*pn*) start replicating their DNA as they migrate toward each other in the zygote. This process can be visualized morphologically as syngamy/ abuttal of *pn* [12]. After DNA replication, the two nuclear envelopes break down, and the 2 *pn* are no longer visible. The zygote subsequently enters the fi rst mitotic division and cleaves and two embryonic cells, or blastomeres, are formed. The process from formation of the cleavage furrow until complete separation of the two daughter cells is denoted fi rst cytokinesis [13 , 14]. The fi rst cleavage cycle is completed with the fi rst division early on “day 2,” 24–29 h after fertilization [15 – 17]. The two embryonic cells divide during the second cleavage cycle, forming a 4-cell embryo on day 2.

The third cleavage cycle results in the formation of an 8-cell embryo on day 3, followed by a fi nal round of cell divisions, before compaction occurs, visualized as obscured intercellular boundaries, and the embryo develops into a morula on day 4. Shortly after the morula stage a fl uid-fi lled cavity develops. This appearance of this cavity, the blastocoel, defi nes the beginning of the early blastocyst stage [10]. This cavity expands until it fi lls most of the embryo (full blastocyst stage). Continued expansion leads to a progressive thinning and, eventually, focal rupture of the surrounding zona pellucida (ZP). Escape of the mammalian embryo from the ZP, referred to as hatching, is initiated on day 5–6 in vitro.

Deviations from the above description of a normal in vitro development are often observed and are of particular interest as they presumably represent underlying abnormalities. An extreme short duration of the fi rst division cycle (the 2-cell stage), referred to as a direct cleavage from one to three cells, is often observed in tri- pronuclear embryos presumably as a result of an excess centriole [18]. Direct

cleavage from one to three cells is however also observed in embryos with a presumed normal chromosomal content where the deviation is associated with a signifi cantly lower implantation rate compared to embryos with a normal cleavage pattern [19 , 20]. Likewise, an aberrant fi rst cytokinesis has been correlated to decreased developmental potential [14]. These studies illustrate the potential benefi ts of characterizing not only optimal division patterns but also deviations from the normal pattern as a single embryo is selected for transfer.

Predictive Algorithms

First to demonstrate the potential of morphokinetic-based predictive models, Wong and colleagues [14] predicted the developmental fate of 4-cell embryos with exceptional sensitivity (93 %) and specifi city (94 %). In this model, a combination of three morphokinetic parameters (duration of fi rst cytokinesis, interval between fi rst and second mitosis, and interval between second and third mitosis) was used successfully to predict blastocyst formation or developmental arrest. More recently, Conaghan and colleagues chose to reevaluate solely these parameters during development of a morphokinetic model for prediction of usable blastocysts (blastocysts selected for transfer or frozen storage on day 5) [13]. In this large study, morphokinetic data from fi ve clinical sites were collected from embryos cultured to blastocyst. No patient and treatment selection criteria were used or restrictions to culture conditions enforced [13]. Notably, the resulting predictive algorithm did not achieve the same sensitivity as Wong et al., but when validated on a large independent dataset it was much better at identifying embryos that were less capable of developing to usable blastocysts than those that did (specifi city of 84.7 %, sensitivity of 38.0 %, PPV 54.7 %, and NPV 73.7 %).

Although blastocyst formation and quality has been used as a measure of embryo viability in a number of morphokinetic studies and confers a number of practical advantages when researching and validating new technologies [21 , 22], the information generated only becomes useful when translated into pregnancy and live birth outcome. Only a few studies have investigated the compatibility between morphokinetic prediction of blastocyst formation and quality and prediction of pregnancy outcome and these studies have demonstrated confl icting results. The aforementioned blastocyst prediction model [13] was subsequently tested on a large combined set of transferred embryos with known clinical outcome [23]. This study demonstrated that the model was somewhat effective with a relative increase of 30 % for implantation in the model-selected group of embryos, but it fell short, as there was a concomitant large rejection of embryos from the test cohort, which actually resulted in pregnancy. This highlights the limitations of predicting blastulation only.

Hlinka et al. [24] showed that only 26.4 % of timely blastocysts resulted in a successful implantation, not surpassing current IVF success rates [24]. Moreover, both Kirkegaard et al. [25] and Chamoyou et al. [26] identifi ed several morphokinetic parameters as signifi cant predictors of high-quality blastocyst development, but these same parameters were unable to discriminate between implanted and unimplanted embryos [25 , 26]. In dramatic contrast, Dal Canto et al. [16] showed that signifi cantly shorter cleavage times from the 2-cell to 8-cell stage were predictive of embryos that develop to blastocysts, expand, and implant [16]. In another study, optimal cleavage stage timings proposed for implantation success have also been successfully shown to identify a large proportion of embryos that develop to blastocysts with good morphology [15]. It would seem that further studies are needed to elucidate the interpretation of these discrepancies and determine if predictive algorithms trained to predict blastocyst development could be used to predict implantation.

The fi rst group to construct a morphokinetic-based model to predict implantation potential developed a hierarchical model that uses both morphological observations and kinetic timings to rank embryos in 10 different categories of descending implantation potential [17]. First, embryos are discarded by a set of exclusion criteria

including poor morphology, direct cleavage from 1 to 3 cells, uneven blastomere size at 2-cell stage, and multinucleation at 4-cell stage. Then timings of three morphokinetic parameters were ordered according to predictive strength: time to 5-cell stage, time interval between second and third mitosis, and time interval between fi rst and second mitosis are used to characterize embryos depending on timings lying in or out of acceptable ranges. These optimal time ranges were defi ned by the timings of 247 implanting and non-implanting embryos that were fi rst subdivided into quartiles and the two consecutive quartiles with the highest number of implanting embryos were then selected as in-range values. Embryos that did not develop within these time intervals were considered out of range. This group suggested that categorization of embryos from high to low implantation potential according to this model was improved when compared to using morphology alone (AUC 0.72 vs. 0.64). Nevertheless no statistical difference in implantation rate was found between embryos in the highest scoring category compared to embryos of highest morphological grade [17]. Subsequently, the same group tested the application of this model to data collected from 10 clinical sites in a larger retrospective study and suggested that a relative improvement to the clinical pregnancy rate of 20.1 % per embryo transfer could be achieved compared to a control group of embryos cultured in conventional incubators and selected solely by static morphological grade [27].

However, this study was not randomized and the improved clinical pregnancy rate could also be explained by better culture conditions supplied in a time-lapse incubator compared with the traditional incubator or selection bias. So far no prospective controlled trial has been published to determine if embryo selection using this timelapse model can improve IVF success rates. The IVI group has recently completed a randomized study. Yet unpublished results report signifi cantly improved ongoing pregnancy rate (51.4 % vs. 41.7 %; p = 0.01) and implantation rate (44.9 % vs. 37.1 %; p = 0.02) for embryos selected using time-lapse criteria compared with selection by standard morphological criteria (Rubio et al. [20]). It has been demonstrated, though, that the tested selection model was not transferable from one clinical setting to another without modifi cations [28], thus underlining the diffi culties in determining universal criteria for optimal division patterns.

Since these studies were published, similar hierarchical models to predict implantation have been described and again quartiles yielding highest number of implanting embryos were used to defi ne optimal time ranges and embryos developing in range have been shown to have higher implantation rates than those embryos developing out of range [29 , 30]. Additionally, several investigators have confi rmed that shorter durations of cell cycles and synchronous divisions of sister blastomeres are strongly predictive of implantation and that prolonged durations in one or more cell cleavage cycles and aberrant cleavage behavior are characteristics of nonimplanting embryos [16 , 20 , 24 , 30]. Most strikingly, abrupt cleavage from one to three cells, defi ned by a short 2-cell duration of <5 h, has been shown in a number

of studies to be a strong negative marker of implantation [17 , 20 , 25]. This abnormal cleavage pattern has largely been unnoticed in static routine observations before the introduction of TLI monitoring. It may be argued that the superior ability of morphokinetic models to identify less viable embryos rather than identify embryos of highest reproductive potential may create the basis for a strategy of time-lapse based embryo selection that will translate into improved clinical outcome. Such an approach will have particular relevance in the setting of single embryo transfer.

Recently, the correlation between timing of kinetic parameters and embryonic aneuploidy, has been the focus of several morphokinetic studies [19 , 29 , 31 – 33].

In the past, morphology and sequential embryo scoring systems have had limited success at identifying aneuploid embryos [34 – 37] and static observation of multinucleation on days 2 and 3 has been shown to have a positive association with aneuploidy and used routinely to deselect embryos [38 , 39]. However, a number of preliminary studies suggest that morphokinetic behavior can be used to increase the probability of selecting euploid embryos without invasive genetic screening.

A number of small studies report possible correlations between timings of early mitotic divisions and embryonic aneuploidy [33 , 40 – 42]. One of these studies

suggests that delayed fi rst and second cleavage divisions and a prolonged transition from the 2- to 4-cell stage were signifi cantly correlated to aneuploidy, in particular multiple aneuploidies [40]. This study also confi rmed that embryos undergoing abrupt cleavage from 1- to 3-cells and 2- to 5-cells are predominately aneuploid. Chavez et al. [33] observed cell cycle parameters for 45 embryos up to the 4-cell stage and found that euploid embryos displayed tightly clustered timings when compared to aneuploid embryos, which had more widely distributed comparative timings. In this study, only 30 % of aneuploid embryos displayed normal timings and these normal timings were determined to predict embryonic euploidy with 100 % sensitivity and 66 % specifi city [33]. Most recently, a much larger study analyzing the chromosomal content of 504 embryos by blastomere biopsy on day 3 and array CGH created a hierarchical model to subdivide embryos into four categories (A–D) according to expected risk of aneuploidy [29]. The two morphokinetic variables used in this algorithm included time interval between 2 and 5 cells (>20.5 h) and duration of the third cleavage cycle (t5–t3) (11–18 h). Embryos categorized according to in- or out of range timings suggested by this model showed a signifi cant decrease in the percentage of normal embryos for each decreasing category (A, 35.9 %, B, 26.4 %, C, 12.1 %, and D 9.8 %; $p < 0.001$). Interestingly, this algorithm was better at predicting blastocyst formation, which was interpreted by the authors as strengthening their fi ndings. The area under the curve was 0.634.

A similar number of time-lapse studies have not identifi ed an association between early cleavage timings and blastocyst aneuploidy as determined by trophectoderm biopsy and 24-chromosome analysis [19 , 43 – 45]. In contrast, one of these studies suggested a simple classifi cation model using timing of initiation of blastulation and timing of full blastulation to classify embryos into high-, medium-, or low-risk categories, with an area under the curve of 0.72 [19]. An assumption that TLI parameters correlate with aneuploidy is hardly justifi ed if the same parameters are not

predictive to implantation potential. When this model was tested on a group of transferred blastocysts ($n = 88$) from un-selected non-PGS IVF patients and related to implantation and live birth outcome, the risk classifi cation was shown to correlate to clinical outcome. Interestingly, the relation was consistent, even when accounting for an important confounding parameter, such as age [31 , 32]. The other signifi cant variable identifi ed to differ, between embryos with multiple aneuploidies only and euploid embryos in the Campbell study, was the time to the start of compaction (tSC) [19]. Several other small studies considering ploidy and morphokinetics have reported peri-compaction and cavitation delays in aneuploid embryos diagnosed by comprehensive chromosome screening methods of trophectoderm biopsies. Montgomery et al. reported that where the duration of compaction was <22 h, fragmented embryos were signifi cantly more likely to produce a euploid blastocyst ($p = 0.009$) compared with embryos with longer compaction periods [46]. Melzer also reported longer duration of compaction in aneuploidy blastocysts compared with euploid, using TLI and blastocyst biopsy techniques ($p < 0.004$) [47]. Delays in later developmental stages were also described by Hong et al. [48]. This group reported longer duration to the start of cavitation in aneuploid embryos. The two signifi cant variables providing some discrimination of aneuploidy risk were the time from fi rst cytokinesis ($p = 0.02$) or from the 5-cell stage ($p = 0.01$) to the onset of cavitation ($p = 0.01$)—when the data were considered in quartiles. Ultimately, morphokinetic-based embryo selection models should focus on healthy euploid live birth as the outcome measure. A promising study of over 200 embryos with known implantation outcome data, which did this, presented an early cleavage algorithm with an area under the curve of 0.8 [49].

Limitations for Model Building: Sensitivity, Specifi city, and Confounders

In summary, a large number of publications confi rm that timing of development does indeed differ between viable and nonviable embryos. The challenge is that most studies show divergent results and that no consensus therefore exists on which

parameters are the most predictive. Only a few publications have offered clinically applicable models of embryo selection [13 , 17 , 31] and these models remain to be validated in randomized trials.

Developing valid time-lapse models applicable to heterogeneous patient populations and in different clinical settings is diffi cult, as multivariate hierarchical selection models [17] have been shown not to be transferable from one clinical setting to another without modifi cations [28]. Similarly, in a hypothetical experiment, where a blastocyst prediction model [13] was applied retrospectively on a large set of transferred embryos, a theoretical increase of 30.0 % in implantation rate for embryos grouped as usable compared with the entire test cohort was demonstrated. Notably, 50.6 % of embryos that were categorized as having a low chance of forming usable blastocyst nevertheless resulted in fetal heart beat [23]. While a part of

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the explanation may be found in heterogeneous patient populations and different clinical settings, it also emphasizes one of the crucial dilemmas in developing diagnostic tests in general—the balance between sensitivity and specifi city. The study very nicely illustrates the risks of defi ning too narrow time intervals for optimal division in order to achieve a high specifi city at the expense of a low sensitivity. It thus underlines the importance of carefully considering that a model must not only provide a substantial increase in implantation, but equally important, that a low rejection rate of viable embryos is secured.

Other plausible explanations for the diverging conclusions on which parameters are most predictive are most likely to be found in the distinct differences in the population of embryos studied, the parameters evaluated, the endpoints chosen, and the differences in treatment-related factors and culture conditions between the studies. Embryos from fertile oocyte donors have been shown to proceed faster through the fi rst cellular divisions compared to embryos from infertile patients [50]. However, it remains unanswered whether the signifi cant differences in age may explain that particular fi nding. In mice, culture in 20 % oxygen signifi cantly delays all stages of embryonic development compared to culture in 5 % oxygen [51] as well as infl uences the embryonic metabolism [52]. In humans, culture in high oxygen appears to delay pre-compaction development [53]. ICSI-fertilized embryos have been reported to display an earlier fi rst cleavage than IVF-fertilized embryos [16 , 54 – 56]. The observed difference most likely originates from a difference in the starting time of registration or oocyte activation [57], and can therefore be overcome by normalization to an early event or durations of events. The difference does, however, complicate the comparison of absolute time points between IVF and ICSI populations which is overcome by using intervals between events.

The choice of medium has been shown to infl uence the cleavage rates for human embryos [58 – 62]. Surprisingly, a recent study did not report any correlation [63], which indicates that the impact may depend on the type of medium and perhaps a combination with other factors as well. Factors relating to the infertility treatment, such as gonadotrophin doses, have been reported to affect timing, with embryos from oocyte donors receiving higher doses of gonadotrophin reaching advanced developmental stages later than those receiving lower doses [64]. Since gonadotrophin doses were presumably administered according to the treatment response of the patient, it could be argued that the fi nding might be correlated with patientrelated factors, such as age and ovarian response which are both interrelated—and correlated to prevalence of aneuploidy—rather than the differences in stimulation per se. Finally, studies on time lapse have been conducted using different time-lapse systems. A difference in technology could potentially infl uence the assessment of the embryo and limit the comparability between studies.

These confounding factors become even more important if the time intervals of optimal division are defi ned too narrowly, as small displacement in timing may result in viable embryos being declared nonviable. Arguably, low sensitivity may be attributed to the infl uence of many of these confounding factors demonstrated to affect embryo kinetics and viability, in particular maternal age, oxygen tension, fertilization method, and culture media, which are rarely considered in the predictive models.

Albeit even controlling for these confounders during collection of morphokinetic data, moderate predictive values were still attained by some predictive models [25].

Future Directions

An increasing number of studies demonstrate that timing of development differs between viable and nonviable embryos, evaluated by blastocyst development, clinical pregnancy, or euploidy. The future challenge is to translate this knowledge into clinically useful models that will improve pregnancy rates after single embryo transfer.

A major challenge is that timing not only refl ects viability but is also infl uenced by patient and treatment-related characteristics, which must be considered when predictive models are developed. How much of the variation in timing that is explained by viability and culture conditions remains to be clarifi ed. The diffi culties in transferring a model from one clinic to another may, however, indicate that one model does not fi t all. Furthermore, concern must be taken for both sensitivity and specifi city.

Several studies have reported that shorter durations of cell cycles and synchronous divisions of sister blastomeres are strongly predictive of implantation while prolonged durations in one or more cell cleavage cycles and aberrant cleavage behavior are characteristic of non-implanting embryos. The superior ability of morphokinetic models to identify less viable embryos by deviations from the normal division pattern, rather than to identify embryos of highest reproductive potential by defi ning optimal division patterns, may indicate that the true potential for TLI lies in de-selection of embryos. The association between poor viability, aneuploidy, and certain TLI patterns supports this. Likewise, attention could profi tably be directed toward identifying normal and abnormal patterns in embryos from the individual patient combined with a traditional evaluation of morphology, rather than focusing exclusively on defi ning time points for optimal division, which most likely vary depending on external factors. This might be combined with broad intervals for timing with suffi cient respect for sensitivity and specifi city, in particular perhaps at the later stages of development.

Confl ict of Interest The authors declare no confl ict of interest.

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human reproduction ORIGINAL ARTICLE *Embryology*

Proposed guidelines on the nomenclature and annotation of dynamic human embryo monitoring by a time-lapse user group

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STUDY QUESTION: Can the approach to, and terminology for, time-lapse monitoring of preimplantation embryo development be uniformly defined in order to improve the utilization and impact of this novel technology?

SUMMARY ANSWER: The adoption of the proposed guidelines for defining annotation practice and universal nomenclature would help unify time-lapse monitoring practice, allow validation of published embryo selection algorithms and facilitate progress in this field.

WHAT IS KNOWN ALREADY: An increasing quantity of publications and communications relating to time-lapse imaging of *in vitro* embryo development have demonstrated the added clinical value of morphokinetic data for embryo selection. Several articles have identified similar embryo selection or de-selection variables but have termed them differently. An evidence-based consensus document exists for static embryo grading and selection but, to date, no such reference document is available for time-lapse methodology or dynamic embryo grading and selection.

STUDY DESIGN, SIZE AND DURATION: A series of meetings were held between September 2011 and May 2014 involving time-lapse users from seven different European centres. The group reached consensus on commonly identified and novel time-lapse variables.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Definitions, calculated variables and additional annotations for the dynamic monitoring of human preimplantation development were all documented.

MAIN RESULTS AND THE ROLE OF CHANCE: Guidelines are proposed for a standard methodology and terminology for the of use time-lapse monitoring of preimplantation embryo development.

LIMITATIONS, REASONS FOR CAUTION: The time-lapse variables considered by this group may not be exhaustive. This is a relatively new clinical technology and it is likely that new variables will be introduced in time, requiring revised guidelines. A different group of users from those participating in this process may have yielded subtly different terms or definitions for some of the morphokinetic variables discussed. Due to the technical processes involved in time-lapse monitoring, and acquisition of images at varied intervals through limited focal planes, this technology does not currently allow continuous monitoring such that the entire process of preimplantation embryo development may be visualized.

WIDER IMPLICATIONS: This is the first time that a group of experienced time-lapse users has systematically evaluated current evidence and theoretical aspects of morphokinetic monitoring to propose guidelines for a standard methodology and terminology of its use and study, and its clinical application in IVF. The adoption of a more uniform approach to the terminology and definitions of morphokinetic variables within this developing field of clinical embryology would allow practitioners to benefit from improved interpretation of data and the sharing of best practice and experience, which could impact positively and more swiftly on patient treatment outcome.

[†] The authors consider that the first two authors should be regarded as joint first authors.

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Key words: embryo assessment / embryo development / morphology / developmental kinetics / time-lapse monitoring

Introduction

Preimplantation embryo development is a dynamic event. Assessment of embryo viability, on the other hand, is commonly based on observations of morphology at pre-defined intervals (ALPHA Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011a,b). The introduction of time-lapse technology enables almost continuous monitoring of embryo development through frequent multiple image acquisitions without potentially compromising viability due to the interruption of culture conditions (Wright *et al.*, 1990; Oh *et al.*, 2007). This technology generates comprehensive information regarding morphology and kinetics of embryo development and facilitates observation of dynamic, and often transient, events occurring between static observation periods. Together, these have been defined as 'morphokinetic' variables (Meseguer *et al.*, 2011).

The potential impact of several confounding factors on morphokinetic variables, such as age (Leibenthron *et al.*, 2012; Hampl and Stěpán, 2013), ploidy (Chavez *et al.*, 2012; Campbell *et al.*, 2013a,b), ovarian reserve (Fréour *et al.*, 2012), infertility indication (Wissing *et al.*, 2012), ovarian response to stimulation (Muñoz *et al.*, 2013), gas composition during *in vitro* culture (Meseguer *et al.*, 2012), culture media (Ciray *et al.*, 2012; Basile *et al.*, 2013), embryo biopsy (Terada *et al.*, 2009; Kirkegaard *et al.*, 2012; Kroener *et al.*, 2012), fertilization method (Lemmen *et al.*, 2008; Cruz *et al.*, 2013), cryopreservation of sperm (Garcia *et al.*, 2012), female body mass index (Bellver *et al.*, 2013) and female smoking habits (Fréour *et al.*, 2013), have been assessed. Strong correlations between embryo kinetics and embryo viability have been demonstrated in various studies (Pribenszky *et al.*, 2010; Wong *et al.*, 2010; Cruz *et al.*, 2011; Meseguer *et al.*, 2011; Azzarello *et al.*, 2012; Campbell *et al.*, 2013b; Chamayou *et al.*, 2013; Herrero *et al.*, 2013; Aguilar *et al.*, 2014). These studies have been challenged by a recent publication, reporting live births following the transfer of blastocysts with 'deviant' morphokinetic profiles (Stecher *et al.*, 2014), which has highlighted the need for IVF clinics to proceed with caution when applying embryo exclusion or selection criteria based on time-lapse literature, mainly due to the fact that, to date, all the studies relating to embryo selection parameters have been retrospective, and randomized control trials are required to establish and confirm morphokinetics parameters which enhance selection of the embryo with greatest implantation potential.

In order to allow the comparison of these morphokinetic variables of embryos within a cohort, between patients and most importantly according to their outcome, such as implantation and live birth, measurements associated with embryo development can be recorded or annotated (automatically or by the embryologist) to allow retrospective analysis. Correct annotation is key in order to exploit reliable information from time-lapse systems and 'almost perfect agreement within and between observers has been reported in a clinic utilizing this technology (Sundvall *et al.*, 2013). Such data can be utilized to build models, or algorithms, which aid prospective embryo selection by distinguishing between the morphokinetic parameters of embryos with known outcomes. For this, standardization of the nomenclature and time of annotations is a prerequisite.

Several clinical time-lapse devices with automatic image capture and software are currently available for IVF practice. The systems vary in several ways including device design (modular within a standard incubator or integrated incubation-camera system), type of microscopy and image acquisition utilized (bright or dark field, single or multiple focal planes and frequency of image collection), culture method (single or group embryo culture) and annotation (automatic, semi-automatic or manual) and in the style of embryo selection software tools available (fixed universal algorithm or customisable software) (Campbell, 2014). Whilst this document aims to provide generic guidelines, some of the parameters, such as those associated with pronuclear morphology and dynamics, described within this document may not be clearly observable with a dark field time-lapse system. Additionally, the ability to record or annotate each parameter defined may be restricted by the device or software available.

The aim of this document is to propose guidelines on the nomenclature of morphokinetic parameters and how and when they should be annotated uniformly. The morphological characteristics of static observations established by ALPHA and ESHRE consortiums were used as reference criteria (ALPHA Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011a,b) and time-lapse publications and abstracts were used as a resource. These parameters trace the development of embryos produced from fresh, or cryopreserved then warmed/thawed oocytes, and follow their development until *in vitro* culture is completed (up to the blastocyst stage). It is recognized that users with specialist interests may choose to annotate in greater detail during particular periods of embryo development and that, for practical purposes, the number of parameters annotated routinely may need to be streamlined by practitioners.

Definitions for dynamic monitoring of human preimplantation embryo development

In the following definitions, '*t*' represents time and references to 'frames' refer to images generated by time-lapse photography during *in vitro* culture (Fig. 1, Supplementary data, Video S1 and Table I). Appearance and fading, of a described variable, are represented by 'a' or 'f' respectively and cell, or episode, number are represented by 'n'.

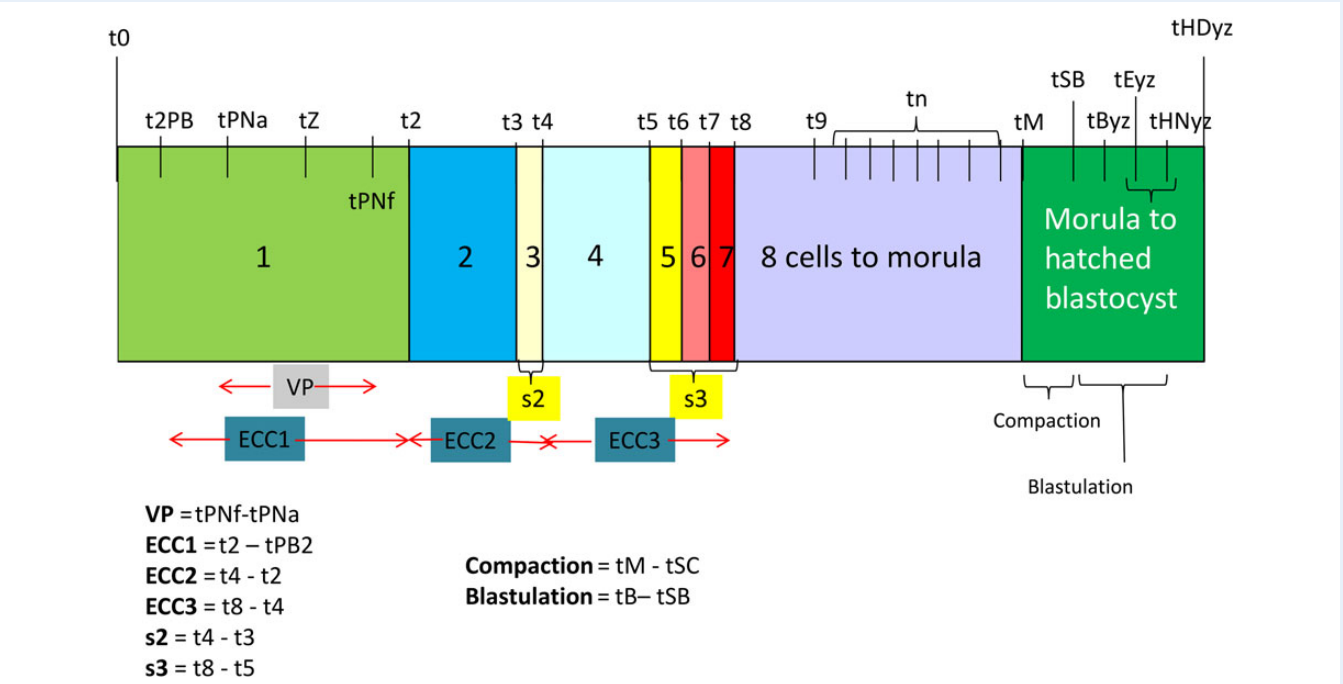

Figure 1 Definitions for the dynamic monitoring of human preimplantation embryo development.

Table I Summary of Morphokinetic variables and proposed definitions.

Timings	
Time	**Definitions of expected events**
t0	Time of IVF or mid-time of micro/injection (ICSI/IMSI)
tPB2	The second polar body is completely detached from the oolemma
tPN	Fertilization status is confirmed
tPNa	Appearance of individual pronuclei; tPN1a, tPN2a; tPN3a..
tPNf	Time of pronuclei disappearance ; tPN1f; tPN2f. . .
tZ	Time of PN scoring
t2 to t9	Two to nine discrete cells
tSC	First evidence of compaction
tMf/p	End of compaction process (last frame before cavity formation) 'f' corresponds to fully compacted; 'p' corresponds to partial compaction
tSB	Initiation of blastulation.
tByz	Full blastocyst (last frame before zona starts to thin) 'y' corresponds to morphology of inner cell mass; 'z' corresponds to morphology of trophectoderm cells
tEyz	Initiation of expansion; first frame of zona thinning
tHNyz	Herniation; end of expansion phase and initiation of hatching process
tHDyz	Fully hatched blastocyst

Each timing defines the first time lapse frame in which the expected phenomenon is observed or detected.

It is recommended that, during manual assessment, time-lapse images are rewound in order to ensure that the correct time point has been selected for the annotation of each variable. In addition, at the outset, users are advised to decide whether non-recording of a variable will indicate its absence or that all variables within the clinic's standard operating procedure will be recorded as absent or present.

t0: The time at which insemination occurs in conventional IVF. For ICSI/IMSI, where the time-lapse monitoring system and practice allows, the time of the sperm injection may be recorded, per oocyte but otherwise, it is the mid-time point from when injection begins and ends for that patient's cohort of oocytes. This time point is used as a start time for the variables below which are measured in hours post insemination/injection, unless otherwise stated.

tPB2: The time at which the second polar body (PB2) is extruded. This is annotated at the first frame in which PB2 appears completely detached from the oolemma. The extrusion of the second polar body can be obscured depending on the position of the oocyte in the well or by cumulus cells in routine IVF insemination.

tPN: The time at which fertilization status is confirmed. It is recommended to annotate fertilization immediately before fading of pronuclei (tPNf) hence coinciding to tZ (time of pronuclear scoring), since no further observational dynamic changes are expected to occur. Appearance of individual pronuclei may be further annotated as tPNna ('n' for individual pronuclei in the order of appearance: 'a'): e.g. tPN1a, tPN2a, tPN3a the initial time at which the first, second, third, etc. pronuclei become visible.

tPNf: The time when both (or the last) PN disappear. This annotation is made at the first frame whereby the embryo is still at the 1-cell stage but pronuclei can no longer be visualized. Pronuclear fading may be further recorded according to individual pronuclei, tPN1f, tPN2f, etc. to

denote the time at which the first, second or additional pronuclei fade (i.e. similar to annotation of their appearances).

tZ: The time of time-lapse PN assessment. PN are dynamic structures; they move and their morphology can change between tPNa and tPNf (Azzarello *et al.*, 2012). It has recently been reported that the movement of the pronuclei within the cytoplasm and fading of nuclear membranes may be indicative of subsequent blastocyst development potential and hence a novel parameter providing an early indication of the embryo's developmental potential (Wirka *et al.*, 2013). Changes in pronuclear appearance and position may coincide with movement of the nucleolar precursor bodies (NPBs) inside pronuclei, allowing differential PN scoring to be deduced. The time-lapse user group recommends annotation of PN scoring, if required, at the last frame before the pronuclei disappear (i.e. tPNf) because the alteration in pronuclear morphology has been completed.

t2: The time of the first cell cleavage, or mitosis. t2 is the first frame at which the two blastomeres are completely separated by individual cell membranes.

t3: The first observation of three discrete cells. The three cells stage marks initiation of the second round of cleavage.

tn: The first time these numbers of discrete cells are observed (until compaction of blastomeres prevents visualization of individual cells).

tSC: The first frame in which evidence of compaction is present; the initial frame that any (two) cells start to compact is observed. The precise timing of initiation of compaction may be difficult to observe due to the increased number of cells and the type of compaction (partial or complete; as described below).

tMf/p: This marks the end of the compaction process; when observable compaction is complete. The morula may be fully or partially compacted, where f is full and p is partial; the morula has excluded material. The degree and time of compaction has been reported to be associated with blastocyst formation and quality (Ivec *et al.*, 2011).

Dynamic developmental stages of blastocyst formation cannot easily be scored using existing static grading schemes (Gardner and Schoolcraft, 1999), for example the time when the blastocoel constitutes less than half the volume of the embryo (early blastocyst) may not be differentiated with certainty from when it is greater than or equal to half of the volume of the embryo (blastocyst). Therefore the time-lapse user group recommends employment of a novel scoring system for depicting the developmental stage of blastocysts, while it is recommended that the morphology of the inner cell mass (*y*) and trophectoderm (*z*) are graded in agreement with the static parameters within the time frame described for dynamic developmental stages, at fixed time points. This group acknowledges that blastomere biopsy may alter the dynamics of embryo development and blastocyst expansion thereby confounding morphokinetic comparisons with non-biopsied embryos (Kirkegaard *et al.*, 2012). However, facilitative laser breaching of the zona pellucida, at the early cleavage stage, to facilitate herniation of trophectoderm for biopsy, has been reported not to impact downstream development to the full blastocyst stage, compared with unbreached controls (Campbell *et al.*, 2013a).

tSB = initiation/start of blastulation. The first frame when initiation of a cavity formation is observed.

tByz = full blastocyst. The last frame before the zona pellucida starts to thin.

tEyz = initiation of expansion. The first frame when the zona pellucida starts to thin.

tHNyz = herniation. The first frame where extrusion of cell(s) from the zona is observed. This marks the end of the expansion phase and the initiation of the hatching process.

tHDyz = hatched blastocyst. The first frame where the embryo is detached from the zona as a whole.

Calculated variables of dynamic monitoring of human preimplantation embryo development

Calculated variables refer to durations of either events to occur or particular morphologies to become visible and they comprise the period in between initiation and termination or appearance and disappearance, respectively (Table II).

Duration of events related to dynamics of early preimplantation period

PN duration (VP: visible pronuclei)

VP is the time period in which the pronuclei are visible. It is calculated as VP = tPNf − tPNa. If pronuclei are annotated individually, the duration for each can be calculated (e.g. tPN1f − tPN1a).

Duration of cell cycles

The cell cycle is an orderly sequence of events in which a cell duplicates its contents and then divides into two (Fig. 2). The duration of the cytoplasmic cleavage and subsequent rearrangements of the individual blastomeres appears to be highly indicative of subsequent viability of embryos (Ramsing and Callesen, 2006). Prolonged cell cycles can be due to DNA repair or cellular rearrangement prior to cleavage (Ramos and de Boer, 2011).

Cell cycle duration is calculated using time-lapse annotation either according to a single cell division or as a round of mitosis whereby the number of blastomeres doubles. For the first cell cycle, as development begins with the single cell, these are the same. However, the second cell cycle begins with two cells, both of which should subsequently divide, forming two daughter cells each. There are therefore two individual blastomere cell cycles but a single embryo cell cycle, which results in the doubling from two to four cells.

Figure 2 provides a schematic to represent the blastomere cell cycles (cc) and the rounds of divisions herein defined as embryo cell cycles (ECC), resulting in the doubling from two to four, and from four to eight, cells. The cell cycle for blastomere 'a' is calculated as t3 − t2 and documented as cc2a, and for blastomere b as t4 − t2, and documented as cc2b. The cell cycle whereby the embryo reaches four cells from two cells (ECC2) is also calculated (t4 − t2). So, the time that the last cleaving blastomere takes to cleave (from t2 to t4) equates to the duration of the ECC; all individual blastomeres cleave within this time frame. The same applies for the third cell cycle. The duration of the embryo's third cycle (ECC3) is the time it takes the embryo to develop from four to eight cells, and includes four blastomere/cell cycles; a, b, c and d. cc3a is t5 − t4; cc3b is t6 − t4; cc3c is t7 − t4 and cc3d is t8 − t4. ECC3 is t8 − t4 (Fig. 3).

An alternative annotation termed 'synchronization', as defined below, is recommended as a simpler alternative:

Table II Summary of calculated variables of dynamic monitoring of human preimplantation embryo development.

	Timings			
Annotations	**Calculated duration of events**		**Dynamic event**	
VP	tPNf-tPNa		PN Duration	
ECC1	t2 − tPB2		Duration of first cell cycle	
ECC2	t4 − t2	cc2a = t3 − t2 cc2b = t4 − t2	Duration of second embryo cell cycle	Duration of single blastomere cell cycle
ECC3	t8 − t4	cc3a = t5 − t4 cc3b = t6 − t4 cc3c = t7 − t4 cc3d = t8 − t4	Duration of third embryo cell cycle	Duration of single blastomere cell cycle
s2	t4 − t3		Synchronization of cell divisions	
s3	t8 − t5		Synchronization of cleavage pattern	
dcom	tMf-tSC (full compaction) tMp-tSC (partial compaction)		Duration of compaction	
dB	tB-tSB		Duration of blastulation	
dexp	tHN-tE		Duration of blastocyst expansion	
dcol	tBCend(n)-tBCi(n)		Duration of blastocyst collapse; 'n' is number of episodes of collapse and re-expansion	
dre-exp	tre-exp end(n)-tre-expi(n)		Duration of re-expansion	
dHN	tHN-tHD		Duration of herniation	

It comprises calculation of events related to dynamics of early and late preimplantation period.

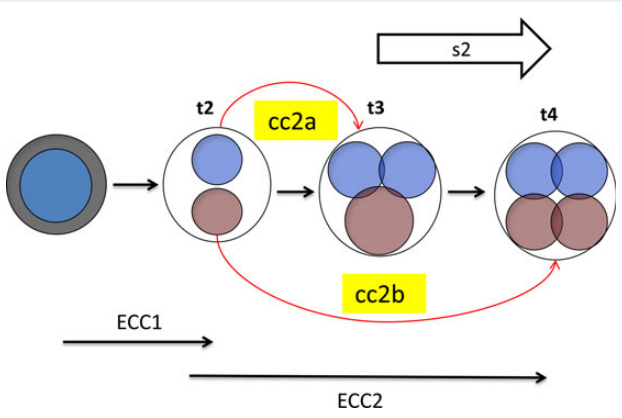

Figure 2 Schematic representation of the blastomere cell cycles (cc) and the rounds of divisions herein defined as embryo cell cycles (ECC), resulting in the doubling from two to four, and from four to eight, cells. The cell cycle for blastomere 'a', is calculated as t3 − t2 and documented as cc2a, and for blastomere b as t4 − t2, and documented as cc2b. The cell cycle whereby the embryo reaches four cells from two cells (ECC2) is also calculated (t4 − t2).

Synchronization

Early embryo development follows a geometric sequence cleavage pattern {1 cell, 2 cells, 4 cells, 8 cells....} as mentioned above, and therefore synchronization can be measured as the time sister cells take to divide into two new cells, reaching the next step in the geometric sequence.

s2 = The synchronicity of the two blastomere divisions within the second cell cycle, calculated as t4 − t3.

s3 = The synchronicity of the four blastomere divisions within the third cell cycle, calculated as t8 − t5.

Duration of cytokinesis (dck)

The duration of each cytokinesis may also be calculated (related to speed of the event and image capture capacity) from the first frame where a cleavage furrow is observed and the time point when cytokinesis is completed.

Duration of events related to dynamics of late preimplantation period

Duration of compaction (dcom)

This is the time period from initiation to cessation of compaction. For full compaction (dcom) = tMf-tSC. For partial compaction (dcom) = tMp-tSC.

Duration of blastulation (dB)

This is the time period from initiation of blastulation to full blastocyst formation (tB-tSB).

Duration of blastocyst expansion (dexp)

This is the time period from initiation of expansion to herniation (tHN-tE).

Duration of blastocyst collapse/re-expansion (dcol/dre-exp)

Regular cycles of expansion and collapse are physiological during blastocyst development (Hardarson *et al.*, 2012). For collapse, these are

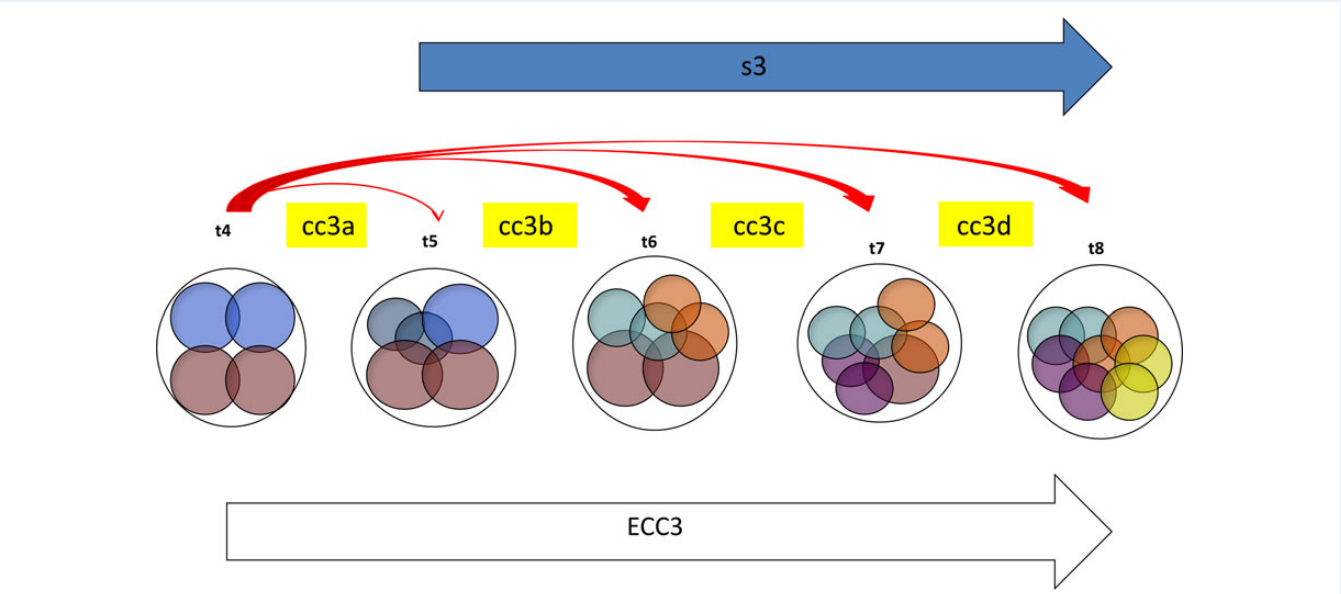

Figure 3 Schematic representation of the third cell cycle. The duration of the embryo's third cycle (ECC3) is the time it takes the embryo to develop from four to eight cells, and includes four blastomere/cell cycles; a, b, c and d. cc3a is t5 − t4; cc3b is t6 − t4; cc3c is t7 − t4 and cc3d is t8 − t4. ECC3 is t8 − t4.

defined as tBCi(n) and tBCend(n), for initiation and completion of the episode, respectively, where 'n' corresponds to the subsequent episodes (for example, tBCi1 = first expansion of blastocyst cavity which follows tBCend1 = first collapse of the blastocoel). The duration of each phase of expansion-contraction cycle can be calculated; the initiation of collapse is annotated according to the first frame in which the blastocoel volume visibly decreased when compared with the volume (diameter) on the previous frame. The final frame prior to the initiation of re-expansion (tBCend) marks the end of the collapse episode. The period in between is the 'duration of collapse (dcol)' and is tBCend(n) − tBCi(n). The initiation of re-expansion is annotated according to the first frame in which the blastocoel volume visibly increased compared with the volume (diameter) on the previous frame. The duration of re-expansion (dre-exp) is tre-expend(n) − tre-expi(n).

Duration of herniation (dHN)

This is the time period from initiation of herniation to hatching (tHN-tHD).

Additional annotations

The proposed annotation for initiation or cessation of the appearance of these events is described below. From these, the duration, or visible period for the event may be calculated as follows: d(event) = t(event)(end) − t(event)(i) (Table III).

Smooth endoplasmic reticulum clusters

Aggregation of smooth endoplasmic reticulum (SER) in oocytes has been associated with suboptimal outcome in some patients (Ebner *et al.*, 2008) (Supplementary data, Video S2). Presence of SER should be annotated as the time points for the appearance, tSER(i) and disappearance, tSER(end).

Fragmentation

To annotate stage-specific fragmentation, *x*%ftn should be used, where *x* is the percentage of fragmentation and tn is the last cell division that was completed. The time-lapse user group suggests the annotation of embryo fragmentation associated with blastomere number, at the final frame prior to the subsequent round of cleavage, because, as development proceeds, embryonic fragments may remain as separate units, or alternatively, may be reabsorbed by the same blastomere from which they were produced or fuse with a neighbouring blastomere (Hardarson *et al.*, 2001; Chavez *et al.*, 2012). For example: '10%ft2' means presence of 10% fragmentation during the final frame 't' at the 2-cell stage.

Annotation of nuclear morphology

nMONO (mononucleated) where 'n' represents the number of blastomeres in which a unique nucleus is seen.

nBI (binucleated) number of blastomeres in which two nuclei per cell are visible.

nMULTI (multinucleated) number of blastomeres in which more than two nuclei are visible. This definition includes micronuclei.

Any cytoplasmic structure without any visible nucleus during the entire cell cycle should be considered as fragments regardless of their sizes.

The time of appearance ('a') and fading ('f') of nuclei can be annotated among individual blastomeres enabling calculation of duration of nuclear visibility. For example, 't4MONO1(a)' depicts the time when the single (mono) nucleus becomes visible in the 'first blastomere' (chosen arbitrarily) of a 4-cell embryo, while t4mono3(f) depicts the fading time of the single nucleus in the third blastomere of the 4-cell embryo.

Blastomere (a)symmetry

Asymmetry of blastomeres is physiological in certain periods of mitotic cycles (e.g. those excluding 2, 4 and 8-cell stages) and such patterns contribute positively to overall embryo selection parameters (Sela *et al.*,

Table III Summary of additional dynamics annotations.

Additional annotations	
Time	**Definitions of special annotations**
devent	Duration of event = t(end)-t(i)
tSER(i)	Appearance of SER
tSER(end)	Disappearance of SER
x%ftn	Stage-specific fragmentation 'x' is percentage of fragmentation 'tn' is last cell division that was completed
nMONO	'n' is number of mononucleated blastomeres
nBI	'n' is number of binucleated blastomeres
nMULTI	'n' is number of multinucleated blastomeres
Even/Uneven cells	Symmetry of blastomere sizes
tTM	Trichotomous mitosis
tFu	Time of cell fusion
tPA	Time of planar blastomere arrangement
tRoll	Time of embryo rolling without divisions
tRoll (i)	Initiation of embryo rolling
tRoll(end)	End of embryo rolling
tCW	Timing of cytoplasmic waves
tCS	Timing of cytoplasmic strings
tV S/M	Presence of vacuoles 'S' represents single vacuole; 'M' represents multiple vacuoles
tG	Appearance of granularity of the cytoplasm

Each timing defines the first time lapse frame in which the expected phenomenon is observed or detected.

2012). Therefore it is recommended that blastomere (a)symmetry is annotated at the end of 2-, 4- and 8-cell stages, during which symmetry of blastomeres is considered optimal morphology. The degree of deviation (i.e. uneven/severe uneven) from normal should be considered as a compromise from embryo viability potentially accompanied with aneuploidy (Hardarson *et al.*, 2001). Cells are described uneven (at the 2-, 4-, 8-cell stages) if blastomeres are more than one third different from sibling cells in size (Puissant *et al.*, 1987). The time-lapse user group encourages annotating alterations in the evenness of blastomeres at these cell stages at the beginning to the end of a particular blastomere division, which may have an impact on subsequent embryo viability.

Irregular cleavage events

Rapid cleavage

Rapid cleavage is a novel dynamic event, which was first described and defined by Rubio *et al.* (2011) as a 'direct cleavage from two to three cells occurring in <5 h'. They have been reported to occur in around 14% of all embryos and they were demonstrated to be one of the most conclusive embryo de-selection parameters, since they compromise implantation capacity (Rubio *et al.*, 2011, 2012).

The time-lapse user group consider that this phenomenon described by Rubio and colleagues is more accurately defined as 'rapid cleavage'. Rapid cleavage describes cleavage of a single cell to two daughter cells occurring faster than the 'normal' duration, which is yet to be defined specific to each cell cycle. This phenomenon may be related to errors in cell cycle mechanisms, which result in this premature cytokinesis. Such irregular cleavage patterns can occur at any cell stage but are most readily identified during early cleavage embryo stage development. As the optimal or normal duration of early cleavage events has not been defined, rapid cleavage cannot yet be annotated as present or not, unless an arbitrary value is introduced. Rather, the durations associated with cleavages can be calculated from the annotation of the embryo reaching sequential cell stages and if and when the normal cell cycle duration is defined, precocious or rapid cleavage can then be identified against normal limits and based on clinical evidence.

Trichotomous mitosis

The first demonstration of, what this proposal is referring to as, trichotomous mitosis was performed and reported by Kola *et al.* (1987) who, employing time-lapse cinematography, described it as an event occurring at the first cleavage of tripronucleated oocytes in order to prevent them developing into triploid embryos (Supplementary data, Video S3). Kola's group considered the spindle forming at the first cleavage in such zygotes, to be tripolar. A year earlier, Angell *et al.* (1986) introduced the concept of direct cleavage and a rapid second division, although they were unable to distinguish between the two irregular phenomena as time-lapse imaging was not employed.

This irregular cleavage event, an aberrant cleavage from a single cell directly to three daughter cells (irrespective of the number of pronuclei), defined as trichotomous mitosis is observable using time-lapse imaging. As in rapid cleavage, this type of irregular division may also occur at any stage of development. Trichotomous mitoses and rapid cleavages can be distinguished from each other as different developmental patterns, possibly associated with differing biological events. Trichotomous mitosis may be associated with errors in spindle apparatus (e.g. tripolar). Trichotomous mitosis, as defined above, can be calculated, for example if $t3 - t2 = 0$, when the embryo did not, at least observably within the acquired images, exist at the 2-cell stage as it cleaved directly to three cells or in a general formula if $t(n) - t(n-1) = 0$. This may be annotated on observation as tTM.

Cell fusion (independent of compaction)

This is defined as a reduction in the number of cells of an embryo during its development due to the merging, or fusion, of cells giving the appearance of a reversed cleavage event (Supplementary data, Video S4). This phenomenon must be distinguished from fragment internalization or re-absorption through identification of a nucleus within the cells involved, prior to occurrence of this event. It is also distinguishable from the merging of cells during compaction preceding morula formation. In an observational study of 1698 zygotes, this phenomenon was observed in 10% of all embryos (Hickman *et al.*, 2012). Although this study demonstrated that cell fusion did not impair embryo development to the blastocyst stage, and was not associated with embryo ploidy, further research is needed to determine the clinical significance of embryos exhibiting such cell fusion, and the mechanisms that cause embryos to undergo this process. When observed, this should be annotated as tFu.

Planar arrangement. Cleavage planes of a 4-cell-stage embryo are considered as normal when they are perpendicularly orientated and blastomeres are tetrahedrally arranged. Some embryos may display

parallel-orientated cleavage axes and are non-tetrahedral or planar possibly due to the disruption of the mitotic spindle (Ebner et al., 2012).

The reported incidence of planar embryos ranges between 3% (Ebner et al., 2012) and 21% (Cauffman et al., 2014). While some associated them with poor implantation capacity (Ebner et al., 2012, Paternot et al., 2014), others reported similar clinical outcomes to tetrahedral embryos (Cauffman et al., 2014). When observed, this may be annotated on observation, tPA.

Embryo rolling. Time-lapse monitoring allows the visualization of embryo rolling; the blastomeres move on themselves without dividing. Prolonged periods of cellular rearrangements following a cell division event can be indicators of poor embryo viability and poor developmental competence as well as poor implantation potential (Ramsing et al., 2007; Cruz et al., 2012). When observed, its beginning and termination should be annotated as tRoll(i) and tRoll(end), respectively.

Cytoplasmic waves. Mouse studies have shown a correlation between rhythmic cytoplasmic movements in oocytes and subsequent development to the blastocyst stage (Ajduk et al., 2011). These movements are caused by contractions of the actomyosin cytoskeleton triggered by calcium oscillations induced by fertilization. Similar waves have been identified in human oocytes; however, they were not correlated with embryo development (Swann et al., 2012). When observed, the waves may be annotated tCW(i) and tCW(end), for their beginning and termination, respectively.

Cytoplasmic strings. Cytoplasmic strings can often be observed in the blastocyst traversing the blastocoel (Supplementary data, Video S5). They are commonly present in early blastocysts and may withdraw as the blastocyst expands. Their persistence in the expanded blastocyst has been associated with poor embryo quality, poor media conditions or a breakdown in polarization (Scott, 2000; Hardarson et al., 2012). More recently, and using time-lapse data from transferred blastocysts, the presence of cytoplasmic strings was observed not to compromise viability (unpublished data).

Time-lapse annotation of the presence or absence of cytoplasmic strings is recommended in order to retrospectively assess their potential value in assessment of embryo viability. When observed, the proposed recommendation for annotation is tCS.

Zona pellucida. The time-lapse user group accepts possible effects on patient or cycle specific basis and proposes annotating exceptional observations regarding the morphology of the zona pellucida. As the appearance of the zona pellucida remains the same throughout the pre-expansion stages, its annotation does not require to be related to time.

Vacuoles. The time-lapse user group proposes the annotation of the presence of vacuoles and the time and duration of appearance. When observed, the proposed guideline for annotation is tV S/M (i) and tV (end), where S refers to a single vacuole; M to multiple vacuoles. As used for other annotations, 'I' and 'end' should be used to annotate the first appearance of vacuoles (i) and when they are no longer visible (end). Measurement of their dimensions may be possible with time-lapse device software tools.

Granularity. Cytoplasmic granularity is poorly defined and its impact on embryo development and viability is not clearly understood. It may be persistent or transitional and should be annotated at the time it is observed and, if applicable, ends. When observed, the proposed guideline for annotation is tG (i) and tG (end).

Discussion

In recent years, the number of studies relating to dynamic development of the preimplantation human embryo development under *in vitro* conditions has increased rapidly and this trend is expected to continue. This paper proposes definitions and annotations for events occurring and observed during dynamic development of the preimplantation human embryo development. During the preparation of this paper, a review paper, incorporating a proposal to standardize nomenclature for time-lapse embryo imaging has been published (Kaser and Racowsky, 2014). While some nomenclature is common, these dedicated guidelines describe and define the dynamic events of preimplantation embryo development more comprehensively, with supporting information and resources. Furthermore, with regard to the important objective of defining clear guidelines in order to maximize its utilization, the current proposal lacks some definitions used by Kaser and Racowsky (e.g. 'formation of expanding blastocyst') since they have been considered too subjective and finite, by this group, for dynamic monitoring as is discussed within. However, others have been highlighted within this document, omitted by Kaser and Racowsky, which have been reported to have clinical significance (e.g. irregular cleavage events).

The major challenge in proposing guidelines for time-lapse technology in IVF arises from the nature and variability of observable events; that they are dynamic. Dynamic events are more difficult to define compared with static observations since the additional dimension of time comes into play. A specific dynamic event may be regarded to occur at its initiation, i.e. the first frame of observation, its end, i.e. that it has been completed, or anytime in between while the event progresses, for example the time when the defined morphology according to static assessments is observed. Examples of such confusions appear as the literature, surrounding morphokinetics of human embryo development, accumulates: pronuclear fading (PNf) as described by this time-lapse user group has been previously defined as the last frame (recorded as tC) where both pronuclei were observed (Chamayou et al., 2013), with a difference of one consecutive frame and as pronuclear disappearance (Lemmen et al., 2008) or pronuclear breakdown (Azzarello et al., 2012). The importance of consensus is enhanced in situations where reference point(s) are established. As an example, time of insemination during ICSI has been defined as 'midway through ICSI' (Campbell et al., 2013a), where other previous studies refer to the time where the procedure has been completed (generally referred as 't0' as in Chamayou et al., 2013). It is of utmost importance that future studies refer to a consented terminology in order to minimize the negative impact of dynamics of these events, and instead provide a benefit gained through this novel technology.

During dynamic monitoring of preimplantation embryo development, some events are easier to observe and define than others. Cleavage-stage cell cycles are such examples since the beginning or termination of dividing blastomeres can clearly be observed through distinct cell membranes unless there is extensive fragmentation. Due to the relative objectivity, ease of study and annotation, early morphokinetic events have been most studied and consequently they feature more frequently, than later events, in algorithms assessing developmental competence

and/or implantation prediction. For example, time between division from two to three cells and from three to four cells have been found predictive in two different algorithms; those described by Wong *et al.* (2010) and Meseguer *et al.* (2011) and referred to as P2 and P3 by the former or cc2 and s2 by the latter groups, respectively. In addition to these parameters, Wong *et al.* (2010) suggested P1, which referred to the duration of first cytokinesis and Meseguer *et al.* (2011), t5 as being the duration for the embryo to reach the 5-cell stage, as an adjunct. In the currently proposed terminology, descriptions suggested in earlier publications have been used as much as possible; however, some have been modified with the intention of simplification and/or clarification. For example, terminology regarding definition of cell cycles has been changed in this proposal; the second cell cycle, as the time the embryo takes to cleavage from two to three cells, is now collectively called CC2, additionally, the time the embryo requires to cleave from three to five cells is called the CC3 (Wong *et al.*, 2010; Meseguer *et al.*, 2011; Herrero *et al.*, 2013; Basile *et al.*, 2014). CC3 was demonstrated as a key parameter associated with greater implantation rates compared with other downstream parameters (Herrero *et al.*, 2013). In this document, the term ECC2 has been proposed, which spans the period where the embryo develops from two to four cells and is divided to CC2a and CC2b, as divisions from two to three and to four cells, respectively. Likewise, the third embryo cell cycle refers (ECC3) to cleavage from four to eight cells and now proposed to be subdivided to CC3a-d in accordance to consecutive blastomere divisions. Because cell cycle terminology and subsequent calculations may be confusing to future investigators, use of terminology regarding synchronization is encouraged. Nevertheless, findings and calculations previously reported, which show a relationship between the duration of blastomeres cell cycles are not invalidated by this proposed terminology.

It is expected that uptake of proposed guidelines for time-lapse terminology will enable utilization of events occurring at later stages of embryo development. Without consensus in terminology and annotation, morula and blastocysts stages of development may be more difficult to track and evaluate, as events are more complex, transitional, overlapping and optically restricted due to the increased number of cellular and a-cellular structures. There are few studies assessing morphokinetics of later development stages yet diversity among terminology and annotations are nevertheless evident. Time of full blastocyst formation has been annotated as tB and described as 'blastocoel filling the embryo with <10% increase in diameter' and time of expanded blastocyst as 'blastocyst increased in diameter by >30% in diameter concomitant to initiation of zona thinning' (Campbell *et al.*, 2013a,b); however, tB has also been defined as the frame in which 'a crescent-shaped area began to emerge from the morula' in the study by Chamayou *et al.* (2013) and the successive frame was the time of expanded blastocyst (tEB), consistent with the 'increase of the overall volume of the embryo and expansion of the blastocoel cavity'. Many of these discrepancies may refer to concepts that are easily understandable. However, the rapid increase in the number of publications concerning time-lapse technology may trigger an endless list of definitions based on the researchers point of view unless a consensus between time-lapse users is not reached.

Through introduction of this novel technology, many events, which had remained obscure with static observation, have come to light. Some examples of such events are abnormal cleavage patterns like trichotomous and reverse cleavages, and rolling of the embryo. Terminology regarding description of these novel events may cause more confusion. For example 'irregular divisions' have been collectively referred to as those from one to three and/or two to five cells in <5 h (Campbell *et al.*, 2013a,b); the former has also been defined as abrupt division (Meseguer *et al.*, 2011), direct (Rubio *et al.*, 2012), tri-polar cleavage (Wong *et al.*, 2010) or very recently as abnormal cleavage (Wirka *et al.*, 2014). A round of mitosis, in the early human preimplantation embryo has been documented to occur 'normally' 10 to 12 h (Cummins *et al.*, 1986). It is yet to be established how much viability may be compromised in those embryos cleaving between 5 and 10 h.

It should be noted that the time-lapse user group does not propose it being necessary to annotate all the parameters listed in this proposal. It is also likely that once the technology becomes available to a wider population of professionals, events that may have escaped current attention will also be described. An update of this current paper will then become a necessity. As stated earlier, the ultimate goal for successful integration of morphokinetic parameters into clinical practise should include: (i) their statistical and biological significance and reproducibility, (ii) their validation through prospective clinical studies proving safety, efficacy and practical utility and (iii) development of reliable technology to measure and quantify such markers (Wong *et al.*, 2013).

Within this rapidly progressing and promising area of reproductive medicine, practitioners now have an additional and increasingly reliable tool for improving embryo selection. The more time-lapse images are interrogated and annotated, and data output is standardized, the more we may understand whether an optimal morphokinetic profile exists. With this, will come the possibility to develop new, and fine tune existing, morphokinetic embryo selection criteria. Time-lapse monitoring is a tool which provides precise information on embryo development, but also has the potential to train, educate and most importantly enhance clinical outcome.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors' roles

H.N.C. played a role in the original conception of the manuscript, and in its design and drafting and gave final approval. A.C. was involved in critical and grammatical revision and drafting of the manuscript and gave final approval. I.E.A. took part in the organizing the meetings, drafted the manuscript, and acquired pictures and videos and gave approval. J.A. was involved in writing the manuscript and critical revision and gave final approval. S.C. took part in the original conception and design of the manuscript and writing of the article and gave final approval. M.E. played a role in drafting and critical revising of article and gave final approval. S.S. played a role in writing the manuscript and critical revision and gave final approval.

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Conflict of interest

H.N.C. is a scientific consultant for ESCO, supplier of Miri TL. I.E.A. is a minor shareholder in Unisense Fertilitech, supplier of the EmbryoScope. Full disclosures of all participants are presented herein. The remaining authors have no conflict of interest.

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12

Noninvasive Techniques: Embryo Selection by Time-Lapse Imaging

Alison Campbell

Introduction

The ability to acquire sequential, photographic, time-lapse images of the developing preimplantation human embryo in vitro has recently provided clinical embryologists with a powerful noninvasive embryo monitoring and selection tool. Time-lapse imaging was used to study fertilization and early human embryo kinetics more than 15 years ago, but this technology is now available for the routine clinical in vitro fertilization (IVF) setting [1]. The first live birth after time-lapse imaging of embryos was reported by Pribenszky and colleagues in 2010, and since then time-lapse imaging of embryos has rapidly become a key topic and area of research in the field of human fertility [2]. This chapter focuses on practical aspects of time-lapse imaging for embryo selection. It covers some of the key findings that have been reported to date and considers the potential impact this exciting technology may have on our understanding of embryo development and on clinical IVF outcome.

Standard versus Time-Lapse Methodology in the Embryology Laboratory

Currently, embryo selection methods rely primarily on morphological evaluation of the embryo based on five or six single, conveniently scheduled observations during their in vitro development. Although these daily observations are recorded and considered, selection of the embryo(s) for transfer, or cryopreservation, tends to be weighted toward the morphology of the embryo just before embryo transfer, focusing on cell number and amount of fragmentation. Selection is often based on a simple embryo grading scheme or a graduated embryo scoring scheme that may also take into account several other variables such as static pronuclear morphology [3,4]. These observations are, in general, not photographic but are recorded by a scribed entry onto a laboratory worksheet, as a database entry, or both. Although this methodology is evidence based, and now guided by a consensus document, time-lapse technology has highlighted the limitations of this rather subjective approach to a crucial clinical decision [5,6].

It is logical that a static snap-shot method to accurately study a dynamic process, such as embryo development, may not be optimal; however, to date, IVF practitioners have worked within these limitations to study and improve embryo selection in the interest of their patients.

A static snap-shot method is not accurate to study a dynamic process such as embryo development.

Conventional light microscopical observations, unlike time-lapse imaging, cannot allow the precise timings of mitoses, or the observation of anomalous cleavage events of the in vitro preimplantation embryo, to be recorded. Furthermore, without time lapse, transient characteristics such as multinucleation can easily be missed when embryos are limited to a single daily observation. Many studies have demonstrated the detrimental effect of multinucleation on embryo implantation, pregnancy, and birth rates [7]. Time-lapse imaging and analysis ensure that such phenomena are observed and recorded, allowing embryo deselection, where appropriate.

Having thousands of time-lapse images available, compared with the usual four to six records of a static observation with conventional methods, may initially seem daunting to the embryologist responsible for embryo selection, but, with robust training and quality assurance, confidence and skills develop quickly and the inquiring mind of the embryologist is opened to this exciting approach.

The wealth of detail that can be observed and information gleaned from such time-lapse images is remarkable. As an example, even before the first cleavage, and within hours of insemination, polar body extrusion can be visualized, providing rapid reassurance that fertilization is likely underway. The dynamics and nature of the proceeding pronuclear formation and morphology now question the extent to which traditional pronuclear grading can assist in embryo selection.

Throughout the preimplantation embryo's development, from insemination to uterine transfer, new phenomena, observed using time-lapse imaging and analysis devices, are being defined and observed as the study of human preimplantation embryology takes on a new direction.

Essential Tools: The Time-Lapse Device

Several clinical time-lapse devices with automatic image capture and software for data collection and analysis are commercially available for IVF practice. Some use the standard incubator (e.g., Primo Vision, Vitrolife, Sweden and Eeva, Auxogyn, United States). Such systems use a single microscope objective within a unit placed inside the incubator chamber to capture images within one culture dish. These systems allow group culture conditions due to the design of the dishes provided with the devices. Each is modular, allowing multiple devices to be fitted within one incubator, with the number depending on the size of the incubator. Primo Vision uses Hoffman contrast integrated optics with green light-emitting diode (LED) (550 nm) illumination and can collect images through 11 focal planes. The Eeva system uses dark-field image capture, in a single focal plane, and automatic cell division tracking and uses software to analyze early embryo development, providing evidence-based quantitative data on each embryo's developmental potential to the blastocyst stage [8]. The Primo Vision system allows user-defined programming of the software to record morphokinetic variables. It also allows published or in-house–developed embryo selection algorithms to be used to rank embryos accordingly. Although widely available, these two systems are not yet cleared for clinical use in the United States.

Selection of a time-lapse system for clinical use is likely to be a task that all IVF centers will be faced with over the next few years to provide the optimum clinical outcome for their patients.

The most widely used integrated device available and in use worldwide is the EmbryoScope (FertiliTech, Denmark). Rather than using a conventional incubator, this device in itself is a nonhumidified incubator with internal circulation of ultraviolet (UV)-light sterilized air through a high-efficiency particulate air (HEPA) and volatile organic compound filtration system. The EmbryoScope has an in-built red LED (635 nm) illumination camera system that arguably allows the most stable and uninterrupted time-lapse imaging and culture. The EmbryoScope can image up to 72 embryos, through a maximum of nine equidistant focal planes. This system includes embryo selection modeling software and provides tools for knowledge building through retrospective analysis of embryo developmental data. A more recently developed device by ESCO Medical (Singapore) is an alternative time-lapse incubation system with capacity for up to 84 embryos from six patients. This system consists of six individually controlled and monitored culture compartments and also requires manual annotation of the acquired time-lapse images.

Selection of a time-lapse system for clinical use is likely to be a task that all IVF centers will be faced with over the next few years to provide the optimum clinical outcome for their patients. It is also likely to be a long-term investment. When deciding which system, or systems, to introduce, consideration should be given to potential impact of clinical outcome, opportunities for development and continuous improvement, device specification, focal planes, image quality and capacity, user friendliness, degree of validation conducted by the supplier and required in-house, certification and licensing (where required), limitations, space, and cost. Customer support, servicing, and training should also be taken into account because accessibility to these may be limited by clinic or supplier location.

Identification and Annotation of Dynamic Embryo Development

When using time lapse, the frequency of image acquisition can take place at variable time intervals set by the individual laboratory. At present 5, 10, 15, or 20 min intervals are most commonly used. As the technology advances, these time intervals may be moved closer, to acquire more detailed information, or even varied during the course of preimplantation embryo development to access more information during certain periods and less during periods of quiescence or where there is evidence of image capture being of lesser clinical impact.

Time-lapse images played, rewound, and paused sequentially as a video can be assessed automatically or semiautomatically by software, or manually. Embryologists and researchers can now study the movement (kinetics) and durations of developmental events alongside embryo morphology. The embryo's developmental patterns and appearance have been referred to as morphokinetics [9]. To date, several specific morphokinetic variables have been correlated to embryo viability, and the outcome measures considered have been blastocyst development, implantation, and live birth. As data from embryos transferred with a known outcome amasses, the most predictive morphokinetic variables for viable embryo selection can be identified. It should be noted, of course, that embryo quality and selection is just one of the several factors influencing IVF treatment success. Factors such as clinical history, embryo transfer procedure, and endometrial receptivity are also of great importance.

Unless the selected time-lapse system has fully automated image capture and analysis tools, some degree of user definability, in terms of morphokinetic variables to be recorded, may be required when establishing a clinical time-lapse service or research program.

Because there may be multiple practitioners in the laboratory involved with the assessment of time-lapse images, it is recommended that key variables for annotation are defined within the standard operating procedure and that these variables are routinely recorded. It is crucial that an early decision is made as to whether variables or morphological features that are not seen in a particular embryo (e.g., compaction or vacuolation) should be reported as "not seen" or whether not reporting them is sufficient to suggest that they were not observed.

Additional or novel variables can be added at a later date once the practitioners have gained familiarity and are able to annotate more swiftly. Annotation is the process of interpreting and recording morphokinetic events on visualizing the time-lapse images and entering this information into the time-lapse device software, where applicable. The introduction of time lapse should not been seen as a burden but rather as a tool that not only dramatically increases flexibility in the embryologists' working day but also has the potential to train, educate, and most importantly enhance clinical outcome. Because time-lapse images viewed as a video can be retrospectively studied and annotated, colleagues can work together to ensure the quality of annotation, discuss queries, and even go back to historical images to annotate new variables at a later date.

Guidelines and consensus are required for standard time-lapse imaging, and to date, without this agreement, several alternative definitions have already been used for the same variable and the approach and standard practice varied from clinic to clinic.

The most commonly used morphokinetic variables are established based on the basic principles of embryology and mitosis and include timing of pronuclear appearance and fading, increasing cell numbers (time to two, three, four, five, six cells, etc.) and times of embryo differentiation to the morula and blastocyst stages. Durations of mitotic cycles and synchronicity, as used in some of the published dynamic embryo selection algorithms, can then be calculated from these variables.

Annotation is the process of interpreting and recording morphokinetic events on visualizing the time-lapse images and entering this information into the time-lapse device software.

If there is a specific research interest, additional "user-defined" variables can be recorded. An example may be surrounding multinucleation. If the number, size, degree, appearance, fading, and dynamics of nuclei are of particular interest during embryo development, this information can be recorded. To facilitate downstream data analysis, it is important that strictly defined terms or phrases are used for these phenomena. An accepted consensus document defining such criteria would assist scientists in data sharing.

Table 12.1 summarizes the commonly used morphokinetic variables and provides basic descriptions and abbreviations for them. The abbreviations in bold may be considered core variables, several of which have been referred to in time-lapse–related clinical publications to date. It is recommended that they are routinely recorded when using time lapse in a clinical setting. This table is not exhaustive and is included as a guideline for users of time lapse. It has been adapted from a document, in preparation, by a team of time-lapse experts aiming to encourage users of time-lapse technologies in IVF to standardize practice for data to be amassed, experiences shared, and best practice reached. Anomalous and non-time-lapse–dependent variables have not been included but may be annotated at the embryologist's discretion and in line with standard operating procedure.

Assurance of Annotation Quality

Ensuring an accurate and objective record of dynamic, and often anomalous, embryo development can be challenging, whether using automatic detection software, the human eye, or even by committee. To amass data of high quality and a resource for embryo selection algorithm development to enhance clinical outcome, a quality assurance system should be introduced early on to ensure that embryologists' or practitioners' interpretations and annotations are objective and consistent. This exercise may be developed in-house or provided by the time-lapse device supplier. Even where there is a sole annotator, data should be compared over time to ensure that consistency and objectivity remain.

Several morphokinetic variables are at risk of subjective interpretation. The appearance of pronuclei and initiation of compaction are just two examples. Thorough training and the use of reference images and audit should be used to ensure annotation quality.

The Challenges of Annotation

Many of the morphokinetic variables within Table 12.1 (see also Figures 12.1 through 12.4) can readily be identified on studying the embryo's time-lapse images, whereas others can cause great discussion and debate. The current lack of an industry standard makes comparing practice and data difficult. An example of one annotation challenge may be fragmentation.

Fragmentation is a poorly understood but a very common and dynamic feature of human preimplantation IVF embryos that has been linked to aneuploidy [10,11]. Time lapse provides an opportunity for the dynamics of fragmentation to be studied in greater detail and has recently been used in a study by Chavez et al. [12] that suggested that most fragmented early cleavage-stage embryos were aneuploid. More recently, a preliminary study by Montgomery et al. [13] has shown an association between the timing and completion of compaction in fragmented embryos, and blastocyst ploidy.

Fragmented embryos with extended periods of compaction were significantly more likely to give rise to aneuploid blastocysts than embryos that completed compaction within 22 hrs of ICSI. Conventionally, fragmentation has been reported by an estimation of the proportion of the embryo affected, and ranges are used rather than specific values [5,6]. To share data and experience with other time-lapse practitioners, surrounding this common feature of human embryos, when annotating fragmentation, it is recommended that a percentage value and the number of cells at the time point of recording are used. There is currently no recommendation for the definition or standard recording of patterning or dynamic movement of fragmentation within the embryo, but there is a desire for such guidelines to be developed.

Outcome Measures

Successful live birth is the ultimate outcome after IVF treatment, and despite the limitations of any outcome measure, including live birth, this should be the primary outcome measure when considering the potential of a developing embryo.

TABLE 12.1

Summary of Morphokinetic Variables and Proposed Definitions

	Description
	Morphokinetic Variables
Time (t)	
t0	IVF or midtime of ICSI/IMSI
tPB2	The second polar body is completely detached from the ooplasm
tnPN	Fertilization status is confirmed
(tPN1a)	The first pronucleus is first visible
(tPN2a)	The second pronucleus is first visible
tPNf	All pronuclei have faded (see Figure 12.1)
t2-t9	Two (see Figure 12.2) to nine sequential, distinguished cells are present
tSC	The first two cells merge; initiation of compaction observed (see Figure 12.3)
tMx/w	Morula is formed or compaction goes no further; "x" corresponds to fully compacted, and "w" corresponds to partially compacted or cells excluded
tSB	The first sign of a cavity is observed as blastulation begins (see Figure 12.4)
tByz	Full blastocyst stage is reached; the last frame before the zona pellucida starts to thin; "y" corresponds to morphology of inner cell mass cells, and "z" corresponds to trophectoderm cells (see Figure 12.4)
tEyz	Initiation of expansion is confirmed; the zona pellucida starts to thin
tHNyz	Extrusion of cells from the zona pellucida is present
tHDyz	Blastocyst is fully hatched from the zona pellucida
	Calculated Variables
VP	tPNf-tPN1a (period of visible pronuclei)
Cell Cycle	
CC1	t2-tPB2 The end of the second meiosis to the formation of two discrete cells
CC2	The time for a two-cell embryo to form a four-cell embryo The two blastomeres (a and b) can be considered individually CC2a = t3-t2 CC2b = t4-t2
CC3	The time for a four-cell embryo to form an eight-cell embryo The four blastomeres can be considered individually CC3a = t5-t4 CC3b = t6-t4 CC3c = t7-t4 CC3d = t8-t4
Synchronization	
S2	The duration of the transition from two sister cells, each dividing to reach the four-cell stage t4-t3
S3	As above, but from four to eight cells t8-t5
Duration of Compaction (Morula Stage)	
tMx-tSC	Full compaction
tMy-tSC	Partial compaction
Blastocyst Stage	
tHN-tSB	Duration of blastulation

Note: Each time point defines the time-lapse frame in which the phenomena described are first observed or recorded.

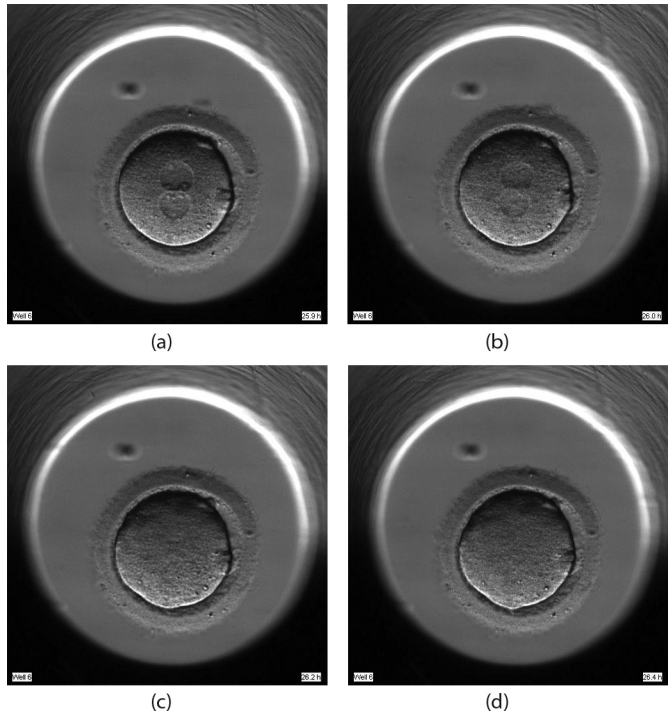

FIGURE 12.1 Sequential time lapse images of fading pronuclei. Image (c) is tPNf.

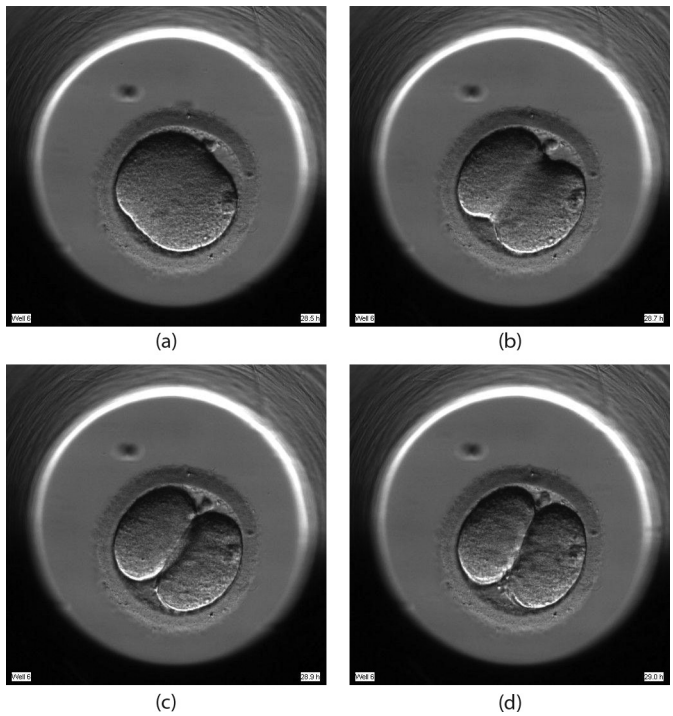

FIGURE 12.2 Sequential time lapse image of the first mitosis. Image (c) shows t2.

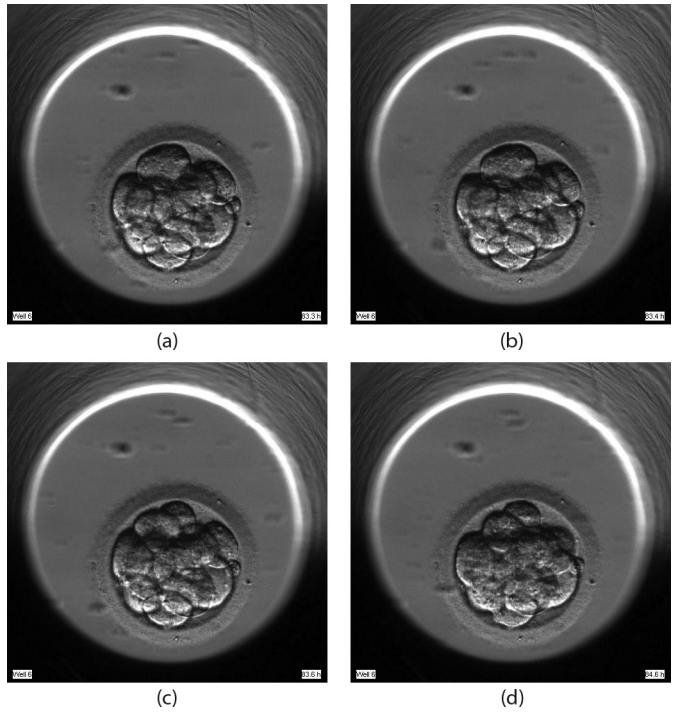

FIGURE 12.3 Initiation of compaction (tSC). More clearly seen with multiple focal planes. Image (c) shows tSC.

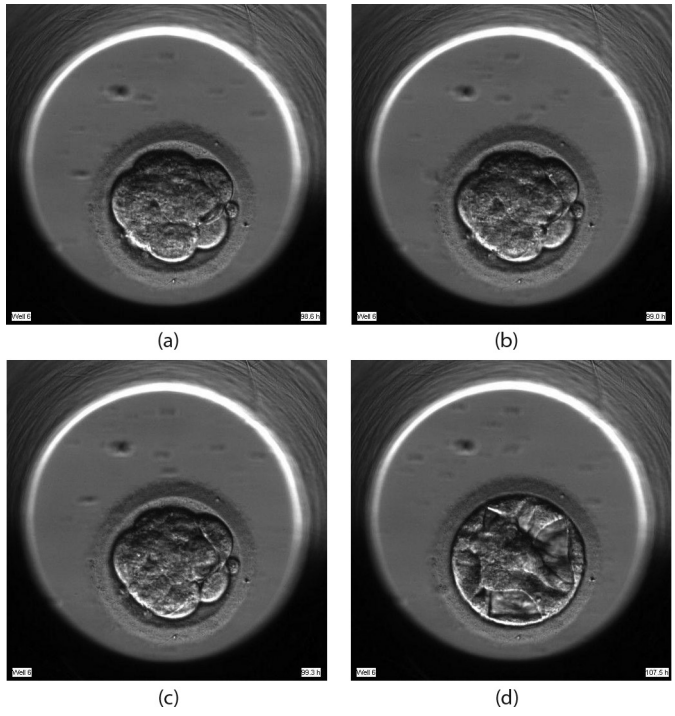

FIGURE 12.4 Initiation of blastulation (tSB). Images (b) and (c) show the increasing cavity and image (d) shows tB.

Due to the relatively recent introduction of time-lapse technology for IVF, the length of the gestational period, and the time required to acquire and collate obstetric outcome data from patients, alternative and less robust outcome measures have been used in published time-lapse studies. Blastulation, although of great scientific interest, has limited clinical value due to the high incidence of blastocysts that fail to implant or are aneuploid [8]. Clinical pregnancy, defined by the presence of a fetal heart on ultrasound scan, has also been used, but due to the early nature of these reported pregnancies, some pregnancies may be lost before term [14]. The difficulty remains that failed pregnancies or even failed implantations cannot necessarily always be attributed to the embryo; however, in terms of outcome measure, live birth arguably remains the most reliable indicator of the viability potential of the preimplantation embryo for IVF and time-lapse practitioners [15].

KID refers to transferred embryos with a known outcome.

What is commonly referred to as "known implantation data," or KID, are the morphokinetic data of a specific and transferred embryo that has a known outcome, with the outcome being either a negative pregnancy test, a gestational sac or fetal heart on ultrasound scan (at 6–8 weeks', gestation), or a live birth. Figure 12.5 describes

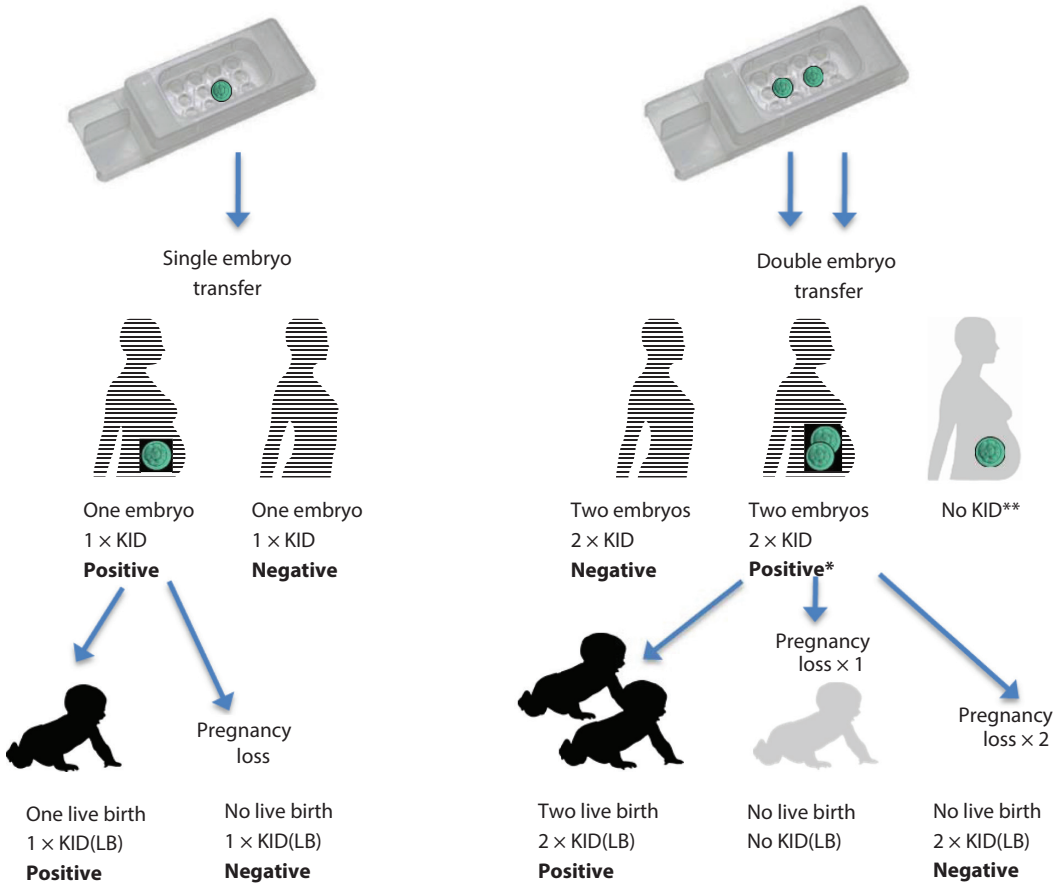

FIGURE 12.5 Known implantation data (KID). KID is the morphokinetic data of a specific, transferred embryo that has a known outcome. *Zygoticity of a twin pregnancy, following double embryo transfer, cannot be ascertained without genetic fingerprinting. Due to relatively low incidence of dizygotic twinning, practitioners, accepting this limitation, may include these data in KID analyses. **As implantation data cannot be deduced or used following a double embryo transfer resulting in a single implantation or birth, the KID ratio, or rate, is lower than the implantation rate per embryo transferred, commonly used for IVF data analyses.

how morphokinetic data may be used for analysis after embryo transfer. Data can be compared between embryos giving positive or negative implantation data (KID+ or KID–, respectively). All data can be used after a single embryo transfer, or a double embryo transfer with a negative outcome. Using data after multiple embryo transfer that has resulted in the same number of fetal hearts or babies born may be problematic without the use of genetic fingerprinting to ascertain the chrorionicity or zygoticity of the pregnancies. However, due to the very low incidence of monozygotic twinning, most analyses could justify the inclusion of these data.

Data Collection and Analysis

Data can be exported from the time-lapse devices for assessment and analysis, or they can be processed using integrated software tools. Provided the data are quality assured and complete, meaning that all relevant morphokinetic variables have been recorded according to standard policy, where they occurred, and that outcome of transferred embryos has been updated, there exists a powerful tool for retrospective analysis. The data should be carefully divided into groups for comparison and statistical analysis. Due to the ranges observed for each morphokinetic variable, it is recommended that median values are used as opposed to means. This way, extreme high or low outliers do not introduce a skew.

KID rates (or ratios) can be calculated for each significant variable for exclusion (deselection) and selection criteria to be identified and then used to develop embryo selection algorithms or models.

KID rates are calculated using the following formula: KID+/KID+ plus KID– × 100%.

Regular data review is recommended to continually improve embryo selection algorithms.

Selection and Deselection Criteria and Algorithms for Embryo Selection

Several time-lapse studies have linked kinetic markers to embryo viability. In a retrospective analysis of EmbryoScope-acquired time-lapse human embryo data, a significant association was previously demonstrated between the timing of pronuclear fading and the first three cleavage events, and successful implantation [16]. Another study, looking at early embryo development, showed an inverse relationship between the ability of embryos to develop to the blastocyst stage and the length of time for zygote division [17].

Time lapse identifies aberrant cleavage patterns.

More recently, a study by Rubio and colleagues [18] focused on the phenomenon of direct, or rapid, cleavage from a single cell to three cells in <5 hr. This study demonstrated the ability of time lapse to identify aberrant cleavage divisions and the reduced implantation potential when such embryos were transferred compared with embryos that did not exhibit this behavior. In a cohort of 1659 transferred embryos, the incidence of this "direct division" was 13.7%, and the known implantation rate of these embryos was statistically significantly lower than for embryos with a normal cleavage pattern (1.2% vs. 20.2%, respectively). Figure 12.6 shows a series of time-lapse images demonstrating this phenomenon within 0.5 hr.

This consecutive series of four time-lapse images, taken at about 10 min intervals, demonstrates the phenomenon of direct cleavage from one to three cells and would not be observed without time lapse and has been reported to be associated with reduced implantation potential [18].

Several embryo selection models, or algorithms, indicating the added value of using morphokinetic information when selecting embryos have now been published. The first by Wong and colleagues [8] identified three significant morphokinetic variables associated with the likelihood of blastulation. These variables and values (in parentheses) were P1, the duration of the first cytokinesis (within 33 min); P2, the interval between the first and the second cytokineses, that is, the total length of time at the two-cell stage (7.8–14.3 hr); and P3, the time between the second and third mitoses, that is, the total length of time at the three-cell stage or the synchrony of cleavage of the first two cells (within 5.8 hr). This work was used to develop the Eeva test (early embryo viability assessment) (Auxogyn).

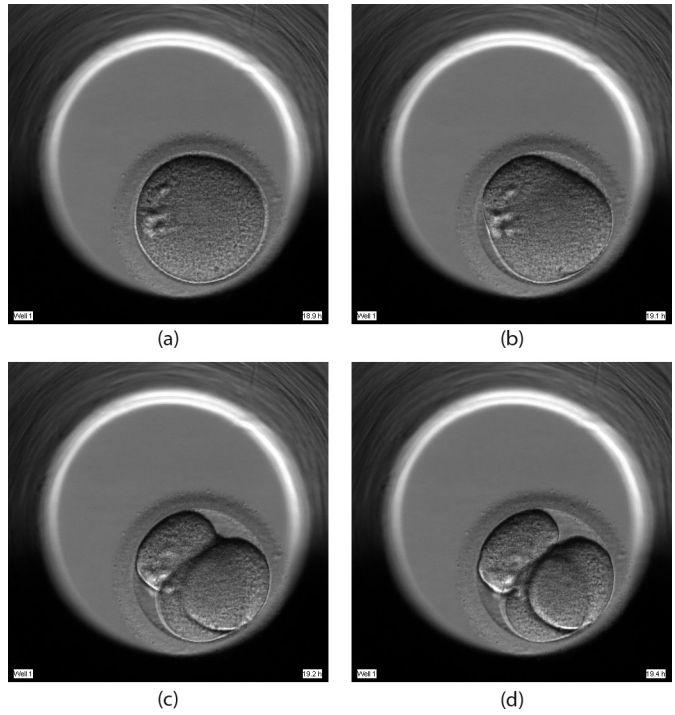

FIGURE 12.6 Rapid or direct cleavage from one to three cells.

The work of Meseguer et al. [9] used the EmbryoScope and looked beyond blastulation to implantation and pregnancy rates. By studying 247 transferred embryos with known implantation outcome, optimal ranges for specific morphokinetic variables were defined. By analyzing multiple preimplantation embryo developmental milestones and durations, a hierarchical model to classify embryos, according to the most significant morphokinetic variables, was developed. The timing of the cleavage to five cells (t5) in this study showed the highest correlation with positive implantation. This model first introduced exclusion criteria. These exclusion criteria were uneven blastomeres after the first cytokinesis, rapid or direct cleavage from one to three cells, and multinucleation at the four-cell stage. After the exclusion of embryos fulfilling these criteria, embryos were classified according to the timings of similar variables proposed by Wong et al. (P2 and P3 above), referred to as cc2 (within 11.9 hr) and S2 (within 0.76 hr), as well as t5 (48.8–56.6 hr post-intracytoplasmic sperm injection [ICSI]). Despite there being some similarities in the morphokinetic variables used within these published models, the timings differ and caution should be exercised when considering applying such models, developed in a different research or clinical setting, before robust validation is performed (as discussed below). It has been demonstrated that a published embryo selection model from one setting may not always effectively be transferred to another setting, but it may be useful with some modification, and after a validation process [19].

Time-lapse–based embryo selection models indicate the value of applied morphokinetics, but care has to be taken while transferring models from one clinical setting to another.

More recently, an aneuploidy risk classification model has been published [20]. Also, using the EmbryoScope, 20 morphokinetic variables and durations were recorded for blastocysts that then underwent trophectoderm biopsy and preimplantation genetic screening (PGS) for all chromosomes using array-comparative genomic

TABLE 12.2

Time-Lapse–Derived Model for Classification of Ploidy with Associated Probabilities of Aneuploidy

Aneuploidy Risk Class	Model	*n*	Probability of Aneuploidy (%)
Low	tB < 122.9 hr and tSB < 96.2 hr	36	37
Medium	tB < 122.9 hr and tSB ≥ 96.2 hr	49	69
High	tB ≥ 122.9 hr	12	97
All		97	61

hybridization (CGH) or single-nucleotide polymorphism (SNP) array. Two variables significantly correlated with embryo ploidy were used to develop an algorithm for embryo selection that classified embryos as having a low, medium, or high risk of aneuploidy. These variables were the start of blastulation (tSB) and the time to reach the full blastocyst stage (tB). Risk classifications were defined according to the time an embryo reached these two developmental milestones. Table 12.2 shows the probability of aneuploidy when embryos were partitioned according to their morphokinetic values for the two significant variables.

This model was later tested retrospectively, by the same researchers, on transferred blastocysts with known clinical outcome that had not undergone biopsy and PGS. Significant relative increases in positive fetal heart and live birth rates were demonstrated when an embryo retrospectively classified as low risk was transferred, compared with the overall rates after blastocyst transfer, indicating the potential clinical applicability of this noninvasive time-lapse algorithm for embryo selection.

Practitioners working with selection models where embryos are deselected or excluded should consider the supporting data to help with the decision process regarding the fate of such embryos. Considerations should be made as to whether the morphokinetic phenomena or timings observed during a particular embryo's development, deeming it deselected for transfer, preclude implantation entirely or are associated with reduced viability or potential. An embryo identified as having a high risk of aneuploidy or low potential to blastulate by a model, for example, may be the highest ranking embryo within a cohort and still give the patient a chance of a positive outcome, albeit small.

Transferability of Embryo Selection Algorithms

Whether such embryo selection algorithms can be directly transferrable between clinics remains to be demonstrated but based on reports that rates of embryo development can differ according to intrinsic or extrinsic factors, it is likely that they may not. Either way, it should be preferable for clinics to develop their own models for their specific patient populations, stimulation regimens, culture conditions, and according to their interpretation of time-lapse images.

Clinics introducing time-lapse methodologies are advised to do so with strict and standard practice throughout the process. Until we know the potential impact of subtle deviations from protocol, culture dishes or slides should be prepared in a standard and precise manner, and time-lapse images, where performed manually, should be assessed objectively and observations recorded (annotated) in the same way by all practitioners. Only this will allow centers to collate robust and complete data to develop in-house embryo selection models that can be fine-tuned as experience and data amasses.

Confounding Factors

There have been several reports on the impact, on embryo morphokinetics, of compounding factors that may be patient or clinic specific. Examples are gas composition during in vitro culture, age, female body mass index, and culture media [14,21–23].

A recent study, however, reported that morphokinetic parameters used for embryo selection were not affected between two different culture media analyzed [24]. Over the next few years, such questions surrounding confounding factors and those crucially asking whether algorithms for embryo selection are transferable will likely be a key focus for time-lapse study in the field of IVF. To make the most effective progress in this field, embryologists are urged to work together, sharing best and common practice to enable this shift in practice to take place most effectively.

Change Control

Due to the possible impact of intrinsic and extrinsic factors on morphokinetic timings, great care should be taken when a change to practice is being considered or made when using time-lapse systems. The impact of a controllable factor such as a change in media or plasticware, for example, on the precise timings of embryo development, should be understood and validated before full implementation, particularly if embryo selection models have been developed under specific conditions. As with all changes made in IVF laboratories, these changes should be justified, controlled, validated, and evaluated but now with the additional consideration of potential impact on morphokinetics, when using time-lapse imaging.

A Tool or a Rule? The Role of the Embryologist

As a clinical treatment, we are still in the relatively early stages of clinical implementation of time-lapse technology. We remain unclear as to whether published algorithms, developed using particular incubation conditions (such as gas mix and culture media), can be directly transferred between centers using not only alternative incubation but also potentially differing definitions and operating procedures for recording dynamic observations of embryo development. Although the expectation is that, eventually, agreement will be reached as to the most significant indicators of an embryo's potential to develop through to live birth, the specific timings may differ according to the variation in, for example, patient history, age, or incubation conditions, and this could result in clinics having numerous algorithms that can be applied for the purpose of embryo selection, according to these factors. In time, and with increasing data and experience, the optimal ranges for defined dynamic events such as the interval between the first and second cytokineses may be further fine-tuned, and additional novel morphokinetic markers of embryo viability will be identified.

The role of the embryologist remains key, despite the most sophisticated algorithm.

The role of the embryologist remains key. Even with sophisticated morphokinetic selection algorithms, the embryologist may need to overrule the algorithm should an embryo reaching all of the milestones within the optimal time ranges also have features considered detrimental, such as smooth endoplasmic reticulum clusters, large areas of cellular degeneration, or late-onset developmental arrest. The use of bright-field time-lapse devices provides embryologists with images already familiar to them and allows this facilitative interaction between the digital and the human eye.

Acknowledgments

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RBMO

ELSEVIER

ARTICLE

‘There is only one thing that is truly important in an IVF laboratory: everything’ Cairo Consensus Guidelines on IVF Culture Conditions

BIOGRAPHY

Participants and speakers at the consensus meeting. Standing from left to right: Jacques Cohen, co-convener and corresponding author, USA; Don Rieger, Canada; Sebastiaan Mastenbroek, the Netherlands; Marius Meintjes, USA; Ronny Janssens, Belgium; James Catt, Australia; Dean Morbeck, New Zealand; David Mortimer, co-convener, Canada; Mohamed Fawzy, program organizer, Egypt. Seated from left to right: Mina Alikani, USA; Sharon Mortimer, writer, Canada; Alison Campbell, UK; Catherine Racowsky, USA; Ragaa Mansour, Egypt. Remote by webinar: Jason Swain, USA.

Jacques Cohen*Cairo 2018 Consensus Group

KEY MESSAGE

This report presents outcomes from an international expert meeting to establish consensus guidelines on IVF culture. Topics reviewed were: embryo culture; temperature; humidity; gas control, pH; workstations; incubators; micromanipulation; handling and assessment; stasis, composition, supplementation, type of culture and storage; equipment and monitoring. More than 50 consensus guideline points were established.

ABSTRACT

This proceedings report presents the outcomes from an international expert meeting to establish consensus guidelines on IVF culture conditions. Topics reviewed and discussed were: embryo culture – basic principles and interactions; temperature in the IVF laboratory; humidity in culture; carbon dioxide control and medium pH; oxygen tension for embryo culture; workstations – design and engineering; incubators – maintaining the culture environment; micromanipulation – maintaining a steady physcochemical environment; handling practices; assessment practices; culture media – buffering and pH, general composition and protein supplementation, sequential or single-step media for human embryo culture; use and management – cold chain and storage; test equipment – calibration and certification; and laboratory equipment and real-time monitoring. More than 50 consensus guideline points were established under these general headings.

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KEYWORDS

Culture conditions
Incubators
IVF
Maintenance
Medium
Quality control

INTRODUCTION

Clinical IVF has had a relatively unrestricted development over the past 40 years, with the result that there is now a plethora of permutations of laboratory culture systems. Some laboratories have retained aspects from the mid-1980s, while others are more likely to embrace change and adopt novel aspects of IVF culture as they are introduced.

Against this background, it is a challenge to identify and define what might constitute 'best practice' in the IVF laboratory. However, there are key physcochemical factors that affect oocytes and embryos in every IVF laboratory: temperature control, maintaining osmolarity and pH, and protection from oxidative stress and toxic substances, such as volatile organic compounds (VOC) (*Mortimer et al., 2018*). The purpose of this consensus workshop, held at the UEARS 2018 conference (17–18 February 2018, Cairo, Egypt) was to define the technical and procedural requirements for an IVF laboratory's culture system while taking these factors into account. The overarching goal of the workshop was to identify how best to operate within a framework of quality and excellence to achieve best practice and to optimize the developmental competency of all gametes received and embryos obtained. The aim was to develop an international expert (TABLE 1) consensus opinion regarding the strengths and weaknesses of various options currently available for equipment and procedures and the criteria by which users can determine fitness for purpose within their own laboratories and operating environments, and to identify areas for priority research to fill knowledge gaps or resolve unknown optimization parameters. It was not the goal to define exactly what should – or should not – be done in the IVF laboratory, as these decisions must be taken in connection with local regulatory and licensing requirements, as well as local availability of products and services.

The structure of the meeting was presentations on the current state-of-the-art aspects of the IVF laboratory culture system, followed by facilitated discussions and the development of a set of guidelines and recommendations for each topic.

WORKSHOP REPORTS

Embryo culture – basic principles and interactions

It is often said that the goal of embryo culture is to replicate the conditions of the maternal reproductive tract. While it is true that the oviduct and uterus provide the best possible culture environment, the reproductive system is extremely complex and is the result of aeons of evolution. Given this complexity, it is unrealistic to suppose that all the diverse interactions between the embryo and the maternal tract will be understood in the near future, and even less realistic to suppose that they can be replicated perfectly *in vitro*. The goal of in-vitro embryo culture is to provide conditions that will lead to the production of embryos that have the same developmental potential as the embryos that develop *in vivo*.

That said, there are at least two facets of the maternal tract that are well recognized and can – and should – be replicated *in vitro*. The first is oxygen tension. Although the exact oxygen tension in the normal human female reproductive tract is not known, it is certain to be well below the ~21% found in ambient air. This has been borne out by the results of perhaps hundreds of studies of the effect of low (5–10%) versus high (ambient) oxygen tension on the development of embryos of every mammalian species studied, including humans. To date, no studies have shown a positive effect of high O_2 on the preimplantation development of human embryos, compared with many that have shown a deleterious effect (see *Gardner, 2016*). Consequently, placing human embryos in a high O_2 environment constitutes exposure to a known embryo toxin, which may be considered unethical.

The second area of concern is the exposure of the embryo to in-vitro conditions with the potential for adverse effects. For instance, it is possible that environmental pollutants, particularly VOCs, may play a role. Although the embryo is not completely protected from VOCs in the maternal tract, the mother's lungs, liver and kidneys do provide considerable filtration and detoxification of VOCs, thus reducing the exposure of the embryo. Conversely, the embryo *in vitro* has no such protective mechanisms, and therefore steps must be taken to actively reduce VOCs in the general laboratory air and within the incubator in particular (discussed in *Mortimer et al., 2018*).

Early approaches to culturing human embryos involved the use of culture media designed for the culture of somatic cells. Many of the components of those media were inappropriate and/or at the wrong concentrations for

TABLE 1 CONSENSUS MEETING PARTICIPANTS AND CONTRIBUTORS

Name	Affiliation
Jacques Cohen[a]	ART Institute of Washington and Althea Science, USA
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Mina Alikani	Northwell Health Fertility of New York, USA
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[b] Reporter.
[c] Coordinator and UEARS representative.

embryo culture. Since then, large strides have been made in the formulations of embryo culture media, beginning with the development of Quinn's Advantage Fertilization (HTF) Medium for culture to the cleavage stages, and then media for culture through to the blastocyst stage. The inclusion of all 20 amino acids has proved to be particularly important, with glutamine being provided via dipeptides with alanine or glycine. Commercial mass-produced and distributed media are manufactured under much more stringent and controlled conditions than could ever be possible in an IVF laboratory, and are subject to rigorous testing and regulatory oversight.

It is, however, important to recognize that the embryo culture medium is but one among perhaps hundreds of factors in the IVF laboratory that might affect the outcome of any given cycle (*Poole et al., 2012*). This includes every pipette tip, holding tube and culture dish that is in contact with any of several media. Laboratory apparatus, gases and procedures could all have effects independent of the culture medium: the fact that a developmental anomaly or failure appears while the embryo is in a culture medium does not mean that the medium is responsible for it. For example, the presence of vacuoles in the sperm head has been shown to have no effect on development through the cleavage stages, but to significantly decrease the rate of development to the blastocyst stage (*Gardner and Kelley, 2017*; *Vanderzwalmen et al., 2008*). There is only one thing that is truly important in an IVF laboratory: everything.

Some consideration must also be given to the measures of success of embryo culture. Although in-vitro evaluation of development can be informative, the evaluation must ultimately include measures of in-vivo development after transfer. Traditionally, this has centred on pregnancy, clinical pregnancy, ongoing pregnancy, miscarriage, birth and live birth rates. None of these are valid measures of embryo viability, because none of them account for the number of embryos transferred. For example, if two embryos are transferred, and one embryo fails to implant in each recipient, it would have absolutely no effect on the clinical pregnancy rate. Clearly, any measure that cannot distinguish between 0% and 50% embryonic loss is of no use in evaluating embryo viability. The use of cumulative pregnancy rate is also inappropriate, because it does not account for the number of transfers. In effect, transfers of viable embryos could be interspersed with the transfer of degenerate embryos, aneuploid embryos, grains of sand, air bubbles, or nothing at all, and it would have absolutely no effect on the cumulative pregnancy rate. Conversely, implantation rate, sustained implantation rate, fetal loss rate, and babies born per embryo transferred all account for the number of embryos transferred. It has been suggested that implantation rate is statistically biased (*Griesinger, 2016*), but this is easily circumvented by doing only single embryo transfer.

Without question, the trend in ART is towards embryo transfer at the blastocyst stage (Days 5–7), and rapidly diminishing transfer at the cleavage stage (Days 2–3). The transfer of cleavage-stage embryos to the uterus is non-physiological, particularly in the presence of elevated oestrogen levels following ovarian stimulation. In addition, culture to the blastocyst stage provides the opportunity for more and better evaluations of development, including morphological, morphokinetic, metabolic and cytogenetic aspects. As a result, the implantation rate for blastocyst transfer is significantly greater than for cleavage-stage transfer for all maternal age groups (*SART National Summary, 2015*–2017). Blastocyst transfer provides better synchrony between the embryo and uterus than does cleavage-stage transfer, so it would seem difficult to justify the transfer of cleavage-stage embryos. In clinics where the current blastocyst development rate might not support this approach, there should be a focus on improving this outcome measure.

However, even with blastocyst transfer, the high concentrations of oestrogen and progesterone resulting from ovarian stimulation produce a less than optimal uterine environment. The logical approach to eliminating this problem is to freeze all of the blastocysts and then transfer them (singly) in one or more subsequent unstimulated cycles (1985c). The implantation rate for the transfer of frozen–thawed blastocysts is significantly greater than for the transfer of fresh blastocysts for all maternal age groups (*SART National Summary, 2016 and 2017*). Moreover, the incidence of small for gestational age (SGA) babies is significantly decreased with frozen embryo transfer, while the incidence of large for gestational age (LGA) babies is significantly increased (*Luke et al., 2017*; *Wennerholm et al., 2013*). It is important to note that SGA is associated with increased overall rates of neonatal, infant and later death but there is no association between LGA and overall rates of neonatal, infant or later death (*Wennerström et al., 2015*; *Xu et al., 2010*).

The incidence of aneuploidy is high, even among morphologically high-quality blastocysts. Such aneuploid embryos have a greatly reduced chance of implanting or of resulting in a live birth. The implantation rate for the transfer of blastocysts identified as euploid by preimplantation genetic testing for aneuploidy (PGT-A) is significantly greater than that for untested blastocysts, for all maternal age groups (*SART National Summary, 2016*). Clinically, the use of PGT-A may be a better standard of care, although accepted evidence is still missing. In terms of experimental design, the presence of aneuploid embryos is a significant source of experimental noise and markedly increases the number of transfers, time to pregnancy, patient distress and cost of hypothesis testing for other aspects of ART.

In conclusion, the basic requirements for embryo culture seem to be:

- complete culture medium (all 20 amino acids);
- attention to all laboratory and clinical factors;
- low O_2 environment;
- low/no VOC environment;
- culture to Day 5(+);
- blastocyst vitrification (freeze-all); and
- optional transfer of single frozen–thawed (euploid) blastocysts.

Temperature in the IVF laboratory

Using and maintaining the appropriate temperature during cell handling and culture is a critical component of optimizing an IVF culture system because improper temperatures can compromise cell function and development and hence reduce IVF outcomes. However, the ideal temperature to use *in vitro* remains an area of debate.

While recreating *in vitro* the conditions that exist *in vivo* to accommodate the embryo's physiological needs is a logical

approach (*Gardner and Leese, 1990*; *Gardner et al., 1996*, *2002*; *Lane and Gardner, 2000a*), there is a risk of errors in the in-vivo measurements (*Ng et al., 2018*). Additionally, the in-vitro environment might necessitate unique considerations and require departure from in-vivo parameters to ensure optimal gamete handling and embryo development *in vitro*.

A core body temperature in humans of 37°C is widely accepted but this value might not be entirely accurate because body temperature is actually a range (*Dewdney, 1993*; *Elert, 2015*; *Mackowiak et al., 1992*; *Sund-Levander et al., 2002*), and can be affected by how the measurement is taken (oral versus axillary versus rectal versus tympanic), the time of day, the individual being measured, and their sex (*Elert, 2015*; *Sund-Levander et al., 2002*). Hence this could bring into question whether the current IVF laboratory standard of 37.0°C is optimal.

Testicular temperature of most mammals, including man, is 2–4°C lower than core body temperature, and is necessary for normal spermatogenesis and spermiogenesis (*Durairajanayagam et al., 2015*). Similarly, the temperature of the female reproductive tract might also be lower than core body temperature: a temperature gradient exists in the oviduct of pigs and rabbits, with the isthmus being 0.2–1.6°C cooler than the ampulla (*Hunter, 2012*; *Hunter and Nichol, 1986*; *Hunter et al., 2006*). Mature follicles in rabbit, pigs and cows are 1.3–1.7°C cooler than the ovarian stroma (*Grinsted et al., 1980*; *Hunter, 2012*; *Hunter and Einer-Jensen, 2005*; *Hunter et al., 1997*, *2000*, *2006*) and ~2.3°C cooler than stroma in human ovaries (*Grinsted et al., 1985*). Such observations, as well as a lack of reliable information on the uterine temperature at the time of implantation, have led to the proposition that human embryo development might benefit from a temperature lower than core body temperature (*Leese et al., 2008*).

Interestingly, apparent detrimental effects of short periods of reduced temperature on the oocyte has led to most practices in the IVF laboratory being performed at a strict 37°C. Prior studies, often using compromised starting material, have shown that: (i) cooling mouse oocytes to near 4°C resulted in zona hardening, probably as a result of premature cortical granule exocytosis; (ii) transient cooling of human oocytes to room temperature caused disorganized spindles, with some oocytes showing displaced chromosomes, and only some of the oocytes showing reorganization of the meiotic spindle upon rewarming; and (iii) the severity of defects increased with lower temperature and longer time (*Johnson et al., 1988*; *Pickering and Johnson, 1987*; *Pickering et al., 1990*). Reassembly of the human oocyte meiotic spindle observed using polarized light microscopy was retarded at room temperatures but was not different between 33°C or 37°C (*Wang et al., 2001*). Additionally, performing intracytoplasmic sperm injection (ICSI) at 37°C, rather than at 33°C or 34°C, resulted in a significantly higher number of oocytes with a visible meiotic spindle, as well as significantly higher fertilization and pregnancy rates (*Wang et al., 2002*).

It should be noted, however, that not all temperature excursions below 37°C are necessarily harmful to the oocyte. One report indicated that it was not necessary to perform ICSI at 37°C for successful outcomes to be achieved (*Atiee et al., 1995*), but did not address the achievement of optimum clinical outcomes. Many successful cryopreservation protocols require equilibration in cryoprotectant solutions at room temperature for several minutes – but these are special circumstances and are associated with careful thawing and warming protocols.

Temperature sensitivity of preimplantation embryos is less well studied. While one study on incubator performance reported an increased pregnancy rate from incubators with a temperature of 36.96 ± 0.13°C compared with those with a temperature of 37.03 ± 0.13°C (*Higdon et al., 2008*), another that reported slight temperature gradients of up to 0.3°C between shelves in culture incubators found no significant impact on clinical outcomes (*Walker et al., 2013*). Of importance, accuracy of these minor temperature variances is less than the uncertainty of measurement of most thermometers. However, a prospective randomized controlled trial (RCT) using patient zygotes cultured for 5–6 days at either 37°C or 36°C demonstrated that culture at 37°C yielded higher average cell numbers on Day 3 and higher blastocyst formation and more useable blastocysts compared with 36°C (*Hong et al., 2014*). Similarly, another prospective study also suggested 37°C was superior for extended embryo culture compared with 36.5°C (*Fawzy et al., 2018*). Thus, prolonged differences in culture temperature appear to impact embryo development.

Current IVF culture media have been formulated for use at ~37°C and it is perceived that alteration of culture temperature could affect cell metabolism as well as medium pH, especially in media containing HEPES or MOPS (*Jeyendran and Graham, 1982*; *Swain, 2010*, *2011*, *2012a*,*b*; *Will et al., 2011*). However, it should be noted that ΔpH/Δ°C is only –0.0096 for 25 mmol/l bicarbonate, hence medium pH barely differs between 36°C and 38°C. Nonetheless, a general change in culture temperature could necessitate medium formulation changes and adjustments to the culture atmosphere.

Although the optimal temperature for culturing embryos might not be established unequivocally, it is critical that a narrow acceptable temperature range be strictly adhered to in order to reduce culture system variability. Therefore, the laboratory should be kept at a temperature that is comfortable for the staff, and equipment set-points should meet the temperature needs of gametes and embryos. This requires the use of calibrated thermometers and proper measurements, i.e. of the culture medium surrounding the oocytes or embryos rather than the surface temperature of equipment (*Cooke et al., 2002*; *Lane et al., 2008*). Regardless, the embryo may experience temperature variations during location changes. Measurements should be performed on a daily basis at a minimum, usually taken in the morning prior to equipment use. Temperature dataloggers are useful in identifying temperature fluctuations during the day and during equipment use. In practical terms, a tightly controlled temperature range is required for each piece of warmed laboratory equipment, although a slightly lower temperature might be safer than a higher temperature.

Humidity in culture

Preimplantation embryos require consistent culture conditions to reduce environmental stress and optimize their development. These conditions include the culture medium and its osmolality and pH (which also depends on the

gas phase composition), temperature, humidity and air quality, all of which are crucial (*Pool et al., 2012*). While there are recommended values or ranges for most of these conditions (*Wale and Gardner, 2016*), the optimum level of incubator humidity has yet to be determined.

Originally, CO_2 regulation in incubators was mediated using thermal conductivity sensors that relied on a humidified atmosphere for correct operation, but the more recent shift to infrared sensors has removed this requirement (*Swain, 2014*). Further, concerns have been expressed that humidity could increase the likelihood of microorganism overgrowth in the incubator, negatively impacting embryo development (*Geraghty et al., 2014*), and it has been postulated that the use of an oil overlay in the culture dishes would prevent evaporation from the culture medium in dry culture, thereby protecting against the risk of increased osmolality. Together, these points have encouraged a shift towards embryo culture in a non-humidified environment.

Two recent studies have demonstrated that an oil overlay is not sufficient to protect against osmolality changes during dry incubation. *Fawzy et al. (2017a)* reported that introducing humidity into a normally dry incubator resulted in improved embryo development and ongoing pregnancy rates. *Albert et al. (2018)* used time-lapse imaging to evaluate the effect of removing humidification from a normally humidified incubator on embryo development, and also reported better outcomes with humidification. It must also be noted that 'humid' versus 'dry' is not a binary condition, and aspects of relative volumes and both surface and interface areas are likely to influence the precise relationship between a humidified incubator and the actual culture environment experienced by the embryos, and *vice versa*. Effects on osmolarity in non-humidified culture may be less deleterious in culture systems with regular medium renewal (every 48 h) as the shift in osmolality accumulates with time (*Fawzy et al., 2017a*; *Swain, 2018*, *Swain et al., 2016*). The central question is why might evaporation occur and osmolarity increase during dry incubation in oil-based culture? Room humidity may also play a role. Considering satisfactory development and outcomes reported in a host of papers after culture in the EmbryoScope™, which is used in a dry environment without medium changeover, one difference may be the large surface area of microdroplets under oil in flat dishes compared with the vertical tube-like culture in first-generation EmbryoScope™ slides. Mouse embryos cultured in non-humidified EmbryoScope™ slides developed as well or better than those cultured in microdroplets under oil in a humidified incubator using the same media (*Kelley and Gardner, 2017*). Until there is clarity regarding the reasons involved in promoting or inhibiting evaporation through oil, embryologists should consider humidification as an obvious choice for culture, but only if incubator manufacturers recommend the use of humidification.

Carbon dioxide control and medium pH

Carbon dioxide control is paramount for culture media to maintain a physiological pH, but it is not as simple as just adding sodium bicarbonate. The pH of a medium is complex, with interactions between amino acids and other zwitterions and proteins (because of their amino acid composition and bound caprylate). Generally, concentrations of CO_2 between 5 and 6% (at sea level) should result in the correct physiological pHe of 7.2–7.4 (*Swain, 2012b*), where pHe is the extracellular pH of the culture medium (*Bavister, 1995*).

But pHe is not the parameter of real biological significance; that is pHi, the intracellular pH of the cytoplasm, which should be about 0.1 pH units below the pHe because the bovine embryo utilizes this gradient to drive transport mechanisms and offset internal acidification from metabolic processes (*Lane et al., 1999*). The pHi of human oocytes and embryos varies slightly during development but is usually accepted to be 7.12, which means the pHe should be 7.2–7.3 (*Phillips et al., 2000*). This pH is achieved by culturing in an environment where the partial pressure of (pCO_2) is titrated to give the correct pH.

In principle, medium pH should be easy to measure, but in practice it is not. There are three main ways to measure the pH of culture media, each with their own pros and cons. A pH meter must have a three-point calibration, ideally inside the incubator. The glass electrode must be kept clean because protein in media will adsorb onto the glass affecting measurement; despite regular cleaning the electrode will still need to be replaced frequently. Blood gas analysers, whether static or portable, can measure pH as well as pCO_2 and pO_2. The results can be accurate but one must be very careful to ensure that the concentration of CO_2 and O_2 is maintained in the gas phase before the measurement is made. But pH meters and blood gas analysers only give a 'snapshot' of the pH, which might change over time.

A new technology uses fluoroscopy to measure pH continuously. It employs immobilized fluorescent dyes that change colour or intensity with the pH. The sensors with standard solutions are placed inside an incubator and give a continual pH readout. The problem with these sensors is that they rely on a standard that might not reflect the pH of the culture medium.

Control of pH is complex, and CO_2 measurement alone is not a good proxy for pHe in a complex culture medium, where pH should be measured directly. Different types and makes of incubators should be thoroughly validated before use, to ensure that the culture medium is maintained at the desired pHe.

Oxygen tension for embryo culture

Another variable important for embryo development and assisted reproductive outcomes is oxygen tension used within the culture incubators. Two alternate levels of O_2 are commonly used *in vitro*: ambient atmospheric O_2 (nearly 21% at sea level) and 5%, the proposed physiological level (review: *Nielsen and Ali, 2010*). Although O_2 levels in the reproductive tract are reported to be between 2 and 8% (*Fischer and Bavister, 1993*; *Kaufman and Mitchell, 1994*; *Kigawa, 1981*; *Mastroianni and Jones, 1965*; *Yedwab et al., 1976*), questions have been raised about the methods used in these studies, prompting a suggestion that they be re-evaluated (*Ng et al., 2018*).

Numerous studies have demonstrated superiority of 5% O_2 compared with ambient O_2 for human embryo culture. For example, better embryo cleavage and blastocyst development *in vitro* under a 5% O_2 atmosphere have been reported, and these culture conditions led to the first IVF live birth (*Edwards*

et al., 1970; Steptoe et al., 1971; Steptoe and Edwards, 1978). Other studies of human embryo culture have reported that 20% O_2 was inferior to 5% O_2 in terms of clinical pregnancy, live birth and implantation (Bontekoe et al., 2012; Meintjes et al., 2009a; Nastri et al., 2016), but recommended further studies with more stringent design and culture protocols. Time-lapse studies of mouse and human embryo culture have shown a detrimental effect of short exposure to atmospheric O_2 (Kirkegaard et al., 2013; Wale and Gardner, 2010).

Oxygen is considered a secondary stressor in embryo culture as it works with ammonium to disrupt the pathway of glutamine and alanine transamination, leading to abnormal fetal development (Wale and Gardner, 2016). Embryo culture under ambient O_2 has also been reported to increase the production of reactive oxygen species (ROS), which can affect embryonic growth and the resulting offspring (Bedaiwy et al., 2004, 2010; Yang et al., 1998). It can also disturb the transcriptome, proteome, carbohydrate and amino acid metabolism, embryo homeostasis, and the epigenome, including inducing premature X-chromosome inactivation, and differentially affects male and female embryos (Gardner and Lane, 2005; Katz-Jaffe et al., 2005; Lengner et al., 2010; Li et al., 2016; Rinaudo et al., 2006; Wale and Gardner, 2012, 2013).

It has been suggested that culture under an even lower O_2 level (2%) from the morula stage onwards might result in the development of healthier embryos, as the uterine O_2 tension is speculated to be lower than that of the oviduct (Ng et al., 2018), and aerobic glycolysis is more important after genomic activation, increasing the risk of ROS production in an O_2-rich environment (Morin, 2017). Studies directly addressing the question of using lower O_2 levels for human embryo culture, by Kaser et al. (2016) and Yang et al. (2016), have been underpowered, although Fawzy et al. (2017b) cultured human embryos continuously from Day 0 to Day 5 or 6 in either 3.5% or 5.0% O_2 concentration and found that while 3.5% O_2 was associated with significantly higher fertilization and cleavage rates it gave significantly lower blastocyst formation and clinical outcomes parameters.

The optimum O_2 level(s) for embryo culture remains to be determined, with the question of whether embryos need stage-specific O_2 levels remaining unclear. For the present, 5% remains the recommended O_2 tension for human embryo culture *in vitro*. One of the surprising facts has been the reluctance of some laboratories to reinvest in reduced O_2 incubators and to add the extra nitrogen gas phase, also associated with increased costs. Financial considerations are a central issue when suggesting guidelines, but the evidence for improved success after introducing reduced O_2 is apparent and must be the prime consideration.

Workstations – design and engineering

The entire IVF process is governed by the biology of the gametes and embryos and it is expected that the laboratory, its design, equipment and operational systems will provide optimal conditions for them. It is incumbent on the laboratory to protect the gametes and embryos from physiological stress (while embryos are highly adaptable, adaptation costs energy and hence equates to metabolic stress) as well as from adverse external factors. Hence, we must select equipment that has been designed and engineered to maintain the correct biophysical and biochemical conditions for each step of the IVF process, and then calibrate and maintain the equipment to ensure it is fit for purpose. Various types of workstation are required in the IVF laboratory so that key tasks can be performed under the appropriate physcochemical conditions: handling and assessing oocytes and embryos; micromanipulation workstations (see next section); processing oocytes and embryos during cryopreservation and thawing/warming; and preparing dishes.

Handling and assessing oocytes and embryos

Although many IVF laboratories use vertical laminar air flow (VLAF) cabinet-based workstations for many or all aspects of oocyte and embryo handling (horizontal LAF cabinets do not provide any protection to the operator), a critical analysis of their ability to support and maintain the key physcochemical factors that affect oocytes and embryos compared with 'IVF chamber' type workstations reveals substantial weaknesses (see Mortimer and Mortimer, 2015).

Optimizing the use of VLAF cabinets as IVF workstations includes accommodating the following challenges:

- While a VLAF-based workstation has very clean air (up to ISO Class 5), it operates at ambient temperature and the high air flow across the work surface causes a substantial cooling effect. The air flow may also enhance evaporation and hence increase osmolality.
- Integral stereozoom microscopes are set into a heated working surface, which must be calibrated to maintain dish contents or drops at 37°C. This can be tricky because although the observation area (glass plate) is surrounded by a heated metal work surface the glass plate is usually heated only by conduction from the sides, and so is cooler.
- Work surface temperature needs to be calibrated for each type of dish that is to be used because different sized dishes have different air gaps that introduce differential insulation.
- When performing dish temperature calibration should the dish lid be on or off, and should the LAF cabinet be running or not? Many laboratories still turn off the air flow when handling open dishes (especially during an egg search procedure), but if the air flow is off then there is no advantage to using a LAF cabinet. Also note that with the lid off (and no oil, e.g. during egg search) the evaporative cooling effect increases as a function of the square of the dish diameter.

Some IVF laboratories use Class II biohazard hoods for oocyte and embryo handling and assessment. While the rationale for this is clear when working with infectious cases (e.g. HIV and hepatitis), the vast majority of laboratories continue to use VLAF cabinets because they work on pre-screened patients.

It has been established that for culture dishes comprising 50 µl droplets of medium under oil, re-gassing (pH re-equilibration) takes ~20 × longer than de-gassing, which is very fast even with oil in that the pH will be >7.4 in <2 min (Debbie Blake, 1999 unpublished data, see Mortimer and Mortimer, 2016). Hence it is recommended that a culture dish be exposed to air for <2 min. Based on these timings, the use of a gas 'hood' (e.g. a glass funnel through which pre-mixed gas is delivered around the dish) can only minimally extend this working time: medium pH will rise, and embryos may be stressed, when the 'out' time exceeds ~2 min, even with rigorous use of a gas hood.

'IVF chamber' workstations have been used since the earliest days of IVF, when they were built using converted neonatal isolettes with a built-in microscope (*Testart et al., 1982*). The internal atmosphere is room air with temperature, CO_2 and humidity control, with contemporary custom-built models including HEPA filtration and even photocatalytic VOC removal. Some manufacturer's chambers have variants that can enclose an entire ICSI rig, and a very recent model has a dual optical system that combines both a stereozoom microscope and a digital inverted microscope with Hoffman-type optics.

How stable could the culture and handling systems be in an ergonomically designed laboratory based on 'IVF chamber' workstations with humidified bench-top incubators? The process would operate as:

- dish removed from bench-top incubator with lid on, so gas environment is maintained;
- dish transferred immediately (5 s) to the workstation;
- lid removed, same pCO_2 inside the workstation;
- lid replaced when finished, enclosing the controlled pCO_2 atmosphere;
- dish transferred (5 s) back into bench-top incubator; and
- bench-top incubator re-equilibrates its internal gas in 30 s.

In all practical terms this would be effectively 'undisturbed culture'. Achieving this using VLAF cabinets will require high operator skills and speed as well as enhanced workstation calibration and quality control (QC). Such cabinets may be recommended as a backup when central HVAC units are out of service.

Processing oocytes and embryos during cryopreservation

Cryopreservation media for both slow freezing and vitrification techniques are usually zwitterion buffered, and hence will be pH stable under an air atmosphere. Processing usually takes place at ambient temperature, although many protocols start or end at 37°C, which can be easily achieved using a heated surface or stage, and by having a 'holding' bench-top incubator nearby (with pre-mixed gas if necessary).

Typically slow freezing and thawing processing was performed in VLAF cabinet-type workstations, but because of the complications of needing liquid nitrogen immediately adjacent to the microscope, and avoiding cold nitrogen vapour accidentally cooling the solutions in the processing dishes, many embryologists perform vitrification and warming procedures on the open bench using a combination of large area (heated) stages on the stereozoom microscope, warming plates, and having a 'holding' bench-top incubator nearby (with pre-mixed gas if necessary).

Dish preparation

This needs to be performed in a VLAF cabinet to maintain sterility of the dishes, culture media and oil during handling, labelling and preparation of the culture dishes. Even though these tasks are all performed at ambient temperature, speed is of the essence to avoid evaporation from microdrops between them being dispensed and overlaid with oil (*Swain et al., 2012*).

Incubators – maintaining the culture environment

The maintenance of incubator performance for each variable within predetermined acceptable limits is, perhaps, the single most important aspect of running an IVF laboratory. Acceptable performance is established through identification of set-points and tolerances. These benchmarks are met after an incubator is turned on for the first time and stabilized, and then are maintained by constant surveillance of performance through a quality management programme. For all variables impacting incubator operation, including temperature as discussed above, gas phase, pH, humidity and air quality, any correction after disturbance in the environment entails an equilibration phase prior to reaching the set-point, and then stabilization within an acceptable range of tolerance (*Swain, 2010*).

Factors influencing stability of the culture environment

There is a variety of factors that influence stability of the culture environment, not the least of which is the frequency of opening/closing the incubator door (if box incubator) or lid (in the case of bench-top incubators). These openings/closings can be kept to a minimum by ensuring there is an adequate number of culture incubators for a programme's caseload, and by having 'holding' incubators dedicated to non-culture activities such as dish equilibrations, sperm preparations, etc. Importantly, only one patient's gametes/embryos should be cultured in any one incubator compartment (whether this is a box incubator with a split door per shelf, or a bench-top incubator). Such a principle not only reduces door opening/closing but also reduces the likelihood of sample mix-up. Beyond physical perturbation of the incubator environment, other factors influencing stability of the culture environment include the type of culture dish, whether a lid is used over the dish, the type and volume of medium used and the protein supplementation, as well as the presence/absence of an oil overlay. Multiple dishes should be prepared in series and not all at once.

Box versus bench-top/top-load incubators offer varying advantages and disadvantages regarding temperature control and measurement and control of humidity (reviewed in *Swain, 2014*). Moreover, the critical requirement for a stable and pure gaseous environment requires consideration of both the supply and control of gases. There are both advantages and disadvantages to using either pre-mixed gas or a gas that is regulated by the incubator. Regardless of which gas supply is used, in-line filters are recommended for removal of various known toxins including VOC, dust, bacteria, etc., and backup gas supplies should be available for use. Individual incubator outcomes should be monitored using key performance indicators such as blastocyst development and pregnancy rates.

Principles for incubator management

There are two broad principles for incubator management:

1. Preventative maintenance, which should be performed at least annually. The machine should be shut down and, if not an incubator that is sterilizable by elevated dry heat, the chamber must be disassembled according to the manufacturer's recommendations. All components, as well as the interior chamber, should be thoroughly cleaned with a diluted mild soap from IVF-specialized manufacturers, although trace residues may be found even after thorough rinsing with deionized water and sterilization with hydrogen peroxide (Fawzy, unpublished data). Hydrogen peroxide can be used

instead of mild soap as it removes protein and other residues. Successful sterilization can be achieved using both diluted mild soap cleaning and sterilization cycles or hydrogen peroxide by itself. Cleaning of time-lapse chambers needs to be performed with great care and manufacturer's instructions must be followed. Chamber ventilation is recommended before the machine is turned back on for recalibration. Aside from cleaning, the gas sensors should be checked against certified standards and the temperature calibrated against a certified NIST thermometer (USA) or performed by an ISO17025 accredited company. A NIST thermometer should be certified within 1 year of use.

2. Daily operation, which should entail implementation of a robust and verified QC programme (see below), minimization of door/lid openings and electrical stability. The latter is particularly important for time-lapse incubators. All incubators should be connected to generator-protected electrical outlets and/or battery operated backup units as well as an alarm system that is regularly tested for functionality and that sends alarms through a telephone tree of personnel to enable quick response.

General rules for incubator QC

Optimum set-points and tolerances should be established for each culture system because different systems probably have different system-specific requirements. These values should be determined using independent calibrated external NIST devices and not be obtained from digital readouts. Each laboratory should determine the minimum frequency for measuring each variable although it is recommended that critical variables discussed previously, such as temperature, CO_2 and O_2, are measured daily, with pH measurements read a minimum of weekly. Recordings should be captured on P-charts (performance charts) or electronically real-time with dataloggers and performance reviewed daily to verify acceptable performance with corrections made as indicated.

Micromanipulation – maintaining a steady physcochemical environment

Micromanipulation includes all procedures requiring technology-mediated distance-controlled robotic manipulation of gametes or embryos. In short, the systems are designed to eliminate tremor and allow precise manipulation of cells, even at high magnification. Micromanipulation rigs are routinely used in all IVF laboratories during ICSI to improve the chances of fertilization, zona opening for enhancing or facilitating hatching and for biopsy of the trophectoderm, and for blastocoel collapse prior to vitrification. Maintaining stable environmental conditions for gametes and embryos during micromanipulation requires the following considerations:

- maintenance of a steady, optimal temperature, similar to culture conditions as described above;
- limitation of exposure to light;
- vibration reduction to reduce damage to cells and enhance ease of handling;
- maintenance of osmolality similar to controlled culture conditions; and
- maintenance of pH similar to controlled culture conditions.

Despite hundreds of papers on work involving micromanipulation, literature on the rationale and technical requirements for achieving steady conditions during micromanipulation is sparse. Consequently, our understanding of optimization, and even the necessity of steady-state conditions, is rudimentary.

Among the most important criteria for maintaining a steady state during excursions of gametes and embryos outside the incubator is the need to avoid potential problems such as unexpected temperature changes or drifts in osmolality. Diligence and risk management should always remain a central focus of an embryology laboratory (see *Mortimer and Mortimer, 2015*, Chapter 9), even when optimization of conditions is relatively unknown.

Intracytoplasmic sperm injection (ICSI)

Sperm preparation methodologies dramatically changed during the first 15 years of clinical IVF. The basic purpose is to remove all traces of seminal plasma and debris and select a subpopulation of highly motile spermatozoa, all in an affordable manner that is fast and with the least trauma to the viable cells. It is known that maintaining physiological pH, temperature and buffering is less critical for spermatozoa than for unfertilized oocytes (*Fleming and King, 2003*), but there are time constraints in terms of exposure to seminal plasma and body temperature (*Cohen et al., 1985a*). Handling techniques should follow general tissue culture techniques and include an appreciation of potential materials toxicity, sperm survival tests, protein supplementation, use of antibiotics, optimal osmolality (250–290 mOsm/kg) and aseptic technique.

Sperm preparation aspects are covered under 'Handling practices' (below). Commercial products include density gradient kits, motility enhancers and hypo-osmotic swelling solutions, and are subject to sterility, pH, endotoxin and osmolarity quality testing. Product QC by the mouse embryo assay (MEA) test is considered essential by some, but its relevance is debatable and some holding products are not MEA tested.

Oocytes are much more sensitive to changes in pH and temperature, and possibly osmolality, than spermatozoa. A deviation from acceptable standards might not affect fertilization but might influence later development. Denudation by hyaluronidase and narrow-bored pipettes or strippers might have long-term effects, but clinical studies are lacking. In 1998, Van de Velde concluded that cytoplasmic maturation must be complete during or shortly after egg retrieval based on an RCT that showed that eggs denuded after 1–2 h had similar outcomes to those denuded at a 4–6 h interval (*Van de Velde et al., 1998*), and this information is used by many practitioners as the basis for ICSI timing – although this will also depend on the interval between ovulation trigger and egg collection. Recently, *Pujol et al. (2018)*, in a large retrospective multivariate analysis, reported that increasing oocyte retrieval to ICSI time increased the fertilization rate, and that each 1 h increase in this interval reduced the likelihood of biochemical pregnancy by 7.3%, and of clinical pregnancy by 7.7% after fresh embryo transfer, although there was no effect on either the ongoing pregnancy or live birth rates. Another large retrospective analysis by *Naji et al. (2018)* reported that retrieval to denudation time intervals of <2 h and 2–5 h did not affect outcomes, but the analysis did not consider the actual trigger-to-stripping interval, and the overall fertilization, implantation and pregnancy rates were modest (68%, 26–27% and 37–38%, respectively).

However, these recent papers contrast with a number of studies published over the last 20 years that generally support the principle that a delay of more than 2 h before oocyte denudation gives improved oocyte maturity, fertilization rate, embryo quality and/or ICSI outcome (*Dozortsev et al., 2004*; *Ho et al., 2003*; *Isiklar et al., 2004*; *Patrat et al., 2012*; *Rienzi et al., 1998*). In addition, another study reported better outcomes by delaying stripping until 4 h post-oocyte retrieval and then performing ICSI promptly, compared with either stripping and injecting immediately after oocyte retrieval or stripping immediately and delaying injection for 4 h (*Hassan, 2001*).

Although clinical information is sparse, prolonged exposure to hyaluronidase could affect oocytes. In the mouse, the duration of hyaluronidase exposure might reduce fertilization and adversely affect subsequent developmental steps (*Ishizuka et al., 2014*). Short co-incubation of spermatozoa and eggs during standard IVF seems to be beneficial according to a Cochrane Review on eight RCT (*Huang et al., 2013*) but the quality of evidence was considered low.

Oocyte handling during micromanipulation

Oocyte handling during regular observations does not involve a change from the standard bicarbonate-buffered conditions, but manipulations such as preparation for ICSI or vitrification could require a change to the external conditions outside the incubator. There is a wide variation in commercially available products for handling oocytes outside incubators: the choice of handling medium is usually HEPES-buffered, less frequently phosphate-buffered saline, while some laboratories still use regular bicarbonate-buffered culture media. MOPS is also used in a number of handling media now. There are heparin-free media as well as antibiotic-free variants for use when patients have allergies. Hyaluronidase is occasionally replaced by other enzymatic products such as recombinant coronase. Sourcing and testing of these products rarely involve studies on fertilization effects or long-term outcomes.

Mechanical stress during micromanipulation

It has been shown that shear stress, similar to the force that occurs during pipetting, has a negative effect on mouse embryos, up-regulating the stress gene for phosphorylated MAPK8/9, formerly known as stress-activated protein kinase/jun kinase/SAPK/JNK (*Xie et al., 2007*); the effects were similar at nearly all stages and enhanced in zona-free embryos. This work is under-appreciated among practitioners and relatively rough handling of gametes and embryos is common in IVF laboratories.

Culture system configurations during micromanipulation

The preferred incubation system during micromanipulation is microdroplets under oil (closed system). Advantages of an oil overlay include: reduced microbial contamination; providing a higher heat capacity and stability than air; controlled separation of fluid droplets; limiting evaporation; and providing a limited VOC sink. ICSI results are probably enhanced when the procedure is performed quickly with a limited number of eggs per dish. There is no recommended maximum egg range per dish, whereas the number of spermatozoa in a pipette should probably be limited. Before starting the procedure, it is recommended to optimize visualization of spermatozoa, egg, pipette tips, alignment of all objects, and perform a quick check of suction devices. The literature is not helpful when defining laser alignment and validation. The use of the blade or laser knife is probably permissible for rapid zona opening, but probably less advantageous when cutting the trophectoderm for biopsy as it might increase cell lysis.

In the mouse, the optimal handling temperature is room temperature and mouse zygotes and embryos are more tolerant to a reduction in temperature during incubation than an increase over 38°C. For human material 37°C is recommended for unfertilized eggs and embryos, and any time at room temperature is a possible confounder. QC assessment of the stage warmer or heating system should be performed periodically, watching for cold and hot spots, drifts and fluctuations.

Polyvinylpyrrolidone (PVP) has been used for half a century in cell culture. For ICSI, PVP (average 10 kDa molecular weight) or an alternative like hyaluronate is used to increase the medium's viscosity so as to decrease sperm motility and reduce fluid movement in micro-pipettes. PVP has been associated with ultrastructural sperm damage and sperm membrane effects, and can delay calcium oscillations and decondensation and remain in the oocyte for an extended time (*Kato and Nagao, 2012*). Half-life time for PVP differs between brands of media (*Kato and Nagao, 2009*).

Handling practices

The use of time-lapse incubators can negate the need to remove embryos from incubators from Day 1 (or Day 0 for ICSI) until the time of embryo transfer. This enables more consistent and possibly better outcomes (*Chen et al., 2017*; *Racowsky et al., 2015*). Unfortunately, there are up to six procedures that must take place during IVF (five for ICSI) before zygotes are put into culture, and up to three procedures at the termination of culture. Care must be taken during these procedures to avoid stressing both gametes and embryos.

General practices

Procedures outside of the incubator usually involve pipetting and microscopic observation. Unless one uses a controlled environmental chamber to conduct these procedures then great care must be exercised to control both temperature and pH. The central importance of these environmental aspects has been described above.

Mathematical modelling and actual measurements of temperature changes during pipetting have shown that a pipette tip quickly loses heat – up to 5°C in 5 s in a glass pipette (*Blomfield, 2011*). Preheating the pipette makes no difference: even though the pipette body retains heat the tip does not. Similarly, keeping embryo transfer catheters in a warm oven does nothing and may increase the VOC concentration inside the catheter, and the catheter tip will be at ambient temperature within seconds. Realization of this rapid loss of heat should govern how embryologists work outside of a controlled environment, and any procedure that is conducted at ambient temperature (e.g. oocyte recovery) should be simulated and a datalogging probe used to determine where any heat losses occur, so they can be minimized.

Most embryologists have been using warm stages for years without fully understanding the basic principles by

which they work. Again, mathematical modelling and actual measurement have shown that, depending on the culture dish and contents, a warm stage takes at least 3 min to start to warm a dish because of the air trapped between the dish bottom and the warm stage. This is exacerbated using metal warm stages with a hole in them.

When one considers that pH changes will show similarities to the temperature changes, then the argument for controlled environment 'IVF chambers' becomes compelling.

Oocyte recovery

A heated tube holder is essential, but precautions should also be taken to minimize heat loss that could occur in either the oocyte retrieval needle or the tubing from the needle to the collection tube (*Yeung et al., 2004*). Simulation measurements (Mortimer and Mortimer, unpublished data) show that the main heat loss is in the oocyte retrieval needle itself and not in the Teflon tubing from the needle to the tube, probably due to Teflon being a much better insulator than stainless steel. The heat loss is variable, being dependent on the flow rate of the fluid, and is minimized when the flow rate is maximal – although flow rate should be restricted to approximately 20 ml/min to avoid damage to the cumulus–oocyte complex (COC). While aspiration of the follicles as quickly as possible is recommended to minimize heat loss, even in a continuous flow model the temperature of the initial part of the aspirate still falls by about 2°C as it enters the collection tube. A possible solution to this problem is to prime the collection tubes with 2 ml of warm buffer to restore the aspirate to 37°C as quickly as possible.

If an environmental chamber is not used, then pipetting during the egg search should be performed as quickly as possible and putative COC should not be held in the pipette while searching for others. Trimming of COC is recommended if blood has infiltrated as this may affect embryo development (*Ebner et al., 2008*). An effect of reducing cumulus size in the absence of blood clots on development has not been shown *Ebner et al., 2017*.

Sperm preparation

Seminal plasma contains one or more factors, prolonged exposure to which adversely affects sperm function, including the ability to penetrate cervical mucus, undergo the acrosome reaction *in vitro* and the fertilization process generally (*Björndahl et al., 2010*; *Mortimer, 2000*; *Yanagimachi, 1994*). Exposure to seminal plasma for more than 30 min after ejaculation permanently diminishes the fertilizing capacity of human spermatozoa *in vitro* (*Rogers et al., 1983*), and contamination of prepared sperm populations with only trace amounts of seminal plasma (0.01% v/v = 1 in 10,000) can decrease their fertilizing capacity (*Kanwar et al., 1979*).

Variables to control during sperm preparation are the choice of method, type of medium and temperature. Currently there are three main methods of sperm preparation: swim-up, microfluidics and density gradients. The first two methods are usually conducted in bicarbonate-buffered 'medium' inside an incubator. Density gradients usually employ a HEPES- or MOPS-based medium ('buffer') for use under air in the centrifuge. The advantage of using 'medium' is that spermatozoa have a requirement for bicarbonate for normal functionality (*Boatman and Robbins, 1991*) and the bicarbonate concentration is reduced in buffer formulations. A 'buffer' is easier to use than a 'medium' because pH control is not a problem.

Temperature control is relatively easy to achieve, but there is some question about what temperature to use. Swim-ups and microfluidic devices are usually inside an incubator so 37°C is the norm, but density gradients are variable depending on the time in the centrifuge, although heated centrifuges are available. Consideration has to be given to these variables and each laboratory will have to define its own protocols to ensure a consistent approach is used. The most common sperm separation techniques have been well described with detailed standardized protocols (e.g. *Björndahl et al., 2010*).

IVF insemination, hyaluronidase use, ICSI and embryo transfer are procedures where pH and temperature can be controlled with an environmental chamber or judicious use of a warm stage and speed. The importance of these aspects was discussed above. To predict the range of temperatures that will occur, each procedure should be simulated, and temperatures checked at key stages using calibrated datalogging temperature probes. Although there will always be a temperature drop during embryo transfer, this can be minimized with care and speed.

Vitrification/warming

There is a plethora of methods and associated commercial media and devices for effective embryo cryopreservation. The most important steps are the initial movement of the embryo from the incubator to the holding buffer, and from the final warming solution to the incubator, because the embryo is more susceptible to temperature changes at these points than in the vitrification solutions, when embryonic metabolism is dramatically slowed. It is suggested that at the first step in vitrification the embryo(s) be removed from culture into the holding buffer at 37°C and allowed to cool to ambient temperature before vitrifying. It is also suggested that if the final warming solution is at ambient temperature then it should be warmed to 37°C before returning the embryos to culture.

Assessment practices

The need to assess

Assessment of gametes and embryos is a necessary, routine aspect of the IVF process, which is primarily morphology based. Gamete assessment aids decision-making, alongside patient clinical factors, regarding the type of fertility treatment required. The frequency and timing of assessment must be specified within a laboratory's SOP and allow sufficient and timely observations to aid effective embryo assessment and selection practice.

Assessment of semen should include, as a minimum, volume, motility, count and morphology of a liquefied sample, following standardized timely procedures and ensuring appropriate temperature control (*Björndahl et al., 2010*; *World Health Organization, 2010*).

Assessment of oocytes can be subjective due to variable morphologies, and the appearance of the cumulus–corona complex or denuded oocyte can be discordant with oocyte maturity or viability. The literature relating oocyte quality and clinical outcome is inconsistent (*Rienzi et al., 2011*). Cumulus removal and oocyte assessment is necessary prior to ICSI, although care

should be taken to ensure this is not performed too soon after oocyte retrieval (as already noted), and the presence of a polar body is the standard requirement to proceed with sperm injection.

Embryo assessment is performed to select the embryo with the highest potential for implantation (*Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011*) and is considered in more detail below. Gamete and embryo assessments allow preferential selection to optimize clinical outcome and are used by embryologists to inform and set expectations for patients, and for monitoring the quality of laboratory performance against relevant key performance indicators (KPI). Laboratory KPI such as fertilization, cleavage, blastulation and embryo utilization rates are considered broad and beneficial indicators of IVF laboratory performance (*ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017*).

Optimizing assessment conditions

Control and maintenance of physcochemical conditions are essential to minimize stress to the gametes and embryos during assessment. During assessments the laboratory environment, equipment and consumables used for handling, as well as the actual manipulations, should be reduced to essential steps only and risks and benefits must be considered when defining related practices. All consumables should be traceable and, where possible, designed and certified for IVF use. Equipment should be preventatively maintained and specified for use. High magnification microscopy should be available (generally 200–400 ×) with Hoffman modulation contrast, or similar, optics being recommended for the detailed examination in plasticware of low-contrast specimens such as oocytes and embryos (*ESHRE Guideline Group on Good Practice in IVF Laboratories, 2016*).

Without time-lapse or video monitoring, assessment of embryos necessitates their removal from the optimized and stable incubator environment. Dedicated or compartmentalized incubators, and models with rapid CO_2 recovery (infrared CO_2 sensors) reduce the possible detrimental impact of inevitable fluctuations in conditions caused by door openings. Oil overlays are recommended to minimize evaporation, gaseous exchange and temperature-induced changes in culture conditions during assessment. As described above, temperature, pH, osmolarity and air quality should be optimized, validated, monitored and standardized across devices and work areas to protect gametes and embryos. Optimal working ranges for these variables should be established and validated for the culture system being utilized. Regular tracking and monitoring of these limits is required for optimized IVF laboratory practice.

The impact of atmospheric or ambient conditions on culture media, and hence the gametes and embryos, must be considered if humidity and gas levels differ between the incubator and the heated assessment area or workstation. General aspects of temperature and humidity during IVF and embryo development were discussed above. With an appropriate heated workstation, the use of zwitterion-buffered media for pH maintenance during very brief visual assessments (<2 min) is not considered justified, although when denuding oocytes and assessing their maturity prior to ICSI such media can be used to provide a stable environment (*Koustas and Sjoblom, 2011*). Medical-grade compressed gases with in-line VOC filters help to ensure high air quality in incubators and workstations, and protect against particulates, VOC and other contaminants. Profiling of pH changes to assess culture media stability limits in the assessment environment is advisable if an isolette/humidicrib-style or similar controlled environment workstation is not available. Exposure to ambient pCO_2 must be controlled and minimized so as to avoid significant changes in medium pH (*Lane et al., 2000*). If sequential culture media are used, then assessments should be aligned with the medium changeover to minimize time outside of stable incubation. For cumulative or sequential embryo assessments over a number of days, single embryo culture or the use of specified microwell culture plates is required to allow robust tracking of individual embryos over time.

The wavelength of light from all sources within the laboratory should be considered if exposure can occur during assessment, and adverse, shorter wavelengths avoided (*Ottosen et al., 2007*). Literature on the relative impact of different wavelengths on human gametes and embryos is sparse, but many IVF laboratories take the precautionary approach of minimizing light exposure and use ultraviolet light filters. The positioning of critical equipment used for assessments is an important consideration during laboratory design: close proximity between the incubators and microscopes helps reduce exposure to ambient laboratory conditions.

Assessment practice and guidelines

Assessment of oocytes and embryos must be performed by properly trained and qualified personnel. Embryologists should be competency-assessed and participate in internal and external quality assurance schemes (*ESHRE Guideline Group on Good Practice in IVF Laboratories, 2016*). Assessments should be performed as swiftly and accurately as possible, for maintenance of intracellular homeostasis, and records of each embryo assessment collected, ideally using live data entry or contemporaneous scribing rather than retrospective recollection. If time-lapse image capture is available, more time is available for the study and assessment of images because the embryos remain within the incubation environment.

Consensus guidelines derived from evidence and experience, and standardized by time of insemination, still provide the most robust foundation for non-invasive gamete and embryo assessment. There are several published grading schemes, offering either formulated or cumulative scoring, or ranking, as well as assessment guidelines from professional organizations (*Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011*; *Cutting et al., 2008*; *Machtinger and Racowsky, 2013*; *Racowsky et al., 2010*, *2011*). However, despite the availability of several guidelines the assessment and selection of embryos is not standardized across the IVF field. Practice varies from single to sequential assessments, to computer-assisted and time-lapse evaluation (*Paternot et al., 2011*; *Racowsky et al., 2009*). Most of the guidelines recommend that cleavage-stage embryo assessment include cell number, size and symmetry, percentage of fragmentation, granulation, vacuoles and nuclear status. Blastocyst stage assessment recommendations include

assessment of expansion, blastocoel cavity size and morphology of the inner cell mass and trophectoderm. Blastocyst grading is most commonly performed using criteria adapted from the scheme published by *Gardner and Schoolcraft (1999a,b)*. Laboratories performing blastocyst culture may use sequential or one-step culture media, the latter with or without continuous culture. Some laboratories use time-lapse observations, others static observations each day or only once or twice. All of these approaches seem to be compatible with acceptable outcomes. The advantages and disadvantages of each of these alternatives are complex and the optimal approach is unknown.

Time-lapse multi-plane imaging provides the opportunity for more detailed and protected assessment, and is generally considered safe practice (*Nakahara et al., 2010*), although oocytes or embryos might need to be removed from this environment to change their orientation or to observe at higher magnification. Time-lapse assessment permits precise logging of cleavage events and developmental milestones; standardized annotation of images is recommended (*Ciray et al., 2014*). Although several time-lapse embryo selection models have been proposed, and while there are similarities between them, they have not been demonstrated to be widely transferable between laboratories (*Barrie et al., 2017*; *Pedersen et al., 2016*).

A recent meta-analysis of five RCT reported an association between morphokinetic embryo selection and significantly higher pregnancy and birth rates compared with standard methodological assessment of embryos cultured in 'big box' incubators, as well as significantly lower early pregnancy loss (*Pribenszky et al., 2017*), while another reported no benefit (*Racowsky and Martins, 2017*). Clearly, further RCT are still needed, as the meta-analyses are of necessity limited by the heterogeneity of the trials included.

Culture media – buffering and pH

Stabilization of pH during IVF is critical to avoid environmental stress that can compromise embryo development. The pH of culture medium, or the external pH (pHe), is normally determined by the balance between CO_2 levels provided in the incubator and, primarily, the sodium bicarbonate concentration in the culture medium, although other factors can impact the final pHe. Periodic fluctuations in culture conditions, such as pHe, can be harmful as these can be transduced into deleterious intracellular perturbations (*Phillips et al., 2000*). Improper intracellular pH (pHi) can impede sperm function (*Babcock and Pfeiffer, 1987*; *Babcock et al., 1983*; *Hamamah and Gatti, 1998*; *Marquez and Suarez, 2007*), impairs embryo metabolism (*Edwards et al., 1998*; *Lane et al., 2000*), alters organelle localization (*Squirrell et al., 2001*), and is detrimental to embryo development (*Lane and Bavister, 1999*; *Lane et al., 1999*; *Leclerc et al., 1994*; *Zhao and Baltz, 1996*; *Zhao et al., 1995*), and even retards subsequent fetal growth (*Zander-Fox et al., 2008*). This is more apparent in cell types like the denuded mature oocyte (*FitzHarris and Baltz, 2006*; *FitzHarris et al., 2007*; *Lane et al., 1999*; *Phillips and Baltz, 1999*), or cryopreserved/thawed embryos (*Lane et al., 2000*), which lack robust pHi regulatory mechanisms, making them especially susceptible to deviations in pHe. Optimizing embryo handling and culture systems must therefore include careful selection of buffers to stabilize pHe.

Buffers are selected based on their optimal pH buffering capacity (the ability to resist pH change) or pKa value, which needs to be matched to the application because many biological processes only function over a narrow range of pHe. Buffers can impact cell development and function independently of pH, and different cell types display different growth rates dependent on the type, as well as concentration, of buffer used (*Eagle, 1971*; *Ferguson et al., 1980*; *Good and Izawa, 1972*; *Good et al., 1966*). Different buffers can also differentially impact various cellular processes, including electron transport, photophosphorylation and mitochondrial oxidation (*Ferguson et al., 1980*; *Good et al., 1966*). Unfortunately, very few comparative studies have been performed to assess the impact of various biological buffers on oocyte and embryo development, but evidence reviewed elsewhere has shown that many concerns regarding the use of buffers other than bicarbonate in human ART are unfounded, being based on misinterpretation of studies with faulty experimental design and invalid conclusions (*Swain and Pool, 2009*; *Will et al., 2011*). From these extensive reviews it can be concluded that:

- Concern over the use of buffers during ICSI on the oocyte's pHi is unwarranted.
- Concerns regarding zwitterionic buffers and IVF are based on studies that were confounded by simultaneous alterations in other media components that impacted embryo development, such as the reduction in bicarbonate levels and the consequent need for less CO_2.
- HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], a modified taurine molecule, is an extensively studied zwitterionic organic buffer, the efficacy of which with spermatozoa is widely accepted; yet its safety with oocytes and embryos remains controversial. This is in spite of numerous studies actually indicating HEPES can efficiently support oocyte maturation (*Byrd et al., 1997*; *Downs and Mastropolo, 1997*), fertilization (*Bhattacharyya and Yanagimachi, 1988*; *Behr et al., 1990*; *Hagen et al., 1991*) and embryo development (*Ali et al., 1993*; *Hagen et al., 1991*; *Mahadevan et al., 1986*; *Ozawa et al., 2006*). In particular, HEPES supports embryo development in the presence of bicarbonate, but not when bicarbonate is absent (*Mahadevan et al., 1986*). Bicarbonate levels influence blastocyst development, possibly through activity of various HCO_3^--dependent transporters, and the CO_2 of the incubator atmosphere is utilized by embryos as a carbon source (*Graves and Biggers, 1970*; *Quinn and Wales, 1971*, *1974*). Indeed, after accounting for CO_2 and bicarbonate levels as co-variables, medium with up to 50 mmol/l HEPES gave similar rates of blastocyst development and cell number compared with medium without HEPES (*Swain and Pool, 2009*).
- MOPS buffer can be used at 25 or even 50 mmol/l to culture mouse zygotes to blastocysts with no significant differences in rates of development or cell number compared with control medium (*Swain and Pool, 2009*).
- Gene expression profiling of environmentally sensitive genes in bovine embryos indicated that expression levels of embryos handled in MOPS or HEPES-buffered media were most similar to embryos derived *in vivo* (*Palasz et al., 2008*).
- MOPS is now included in commercial handling media and used successfully for human ART procedures, ranging from sperm washing to oocyte and embryo handling and vitrification.

MOPS has been reported to be superior to HEPES for vitrification, although the exact reason for this remains unclear (*El-Danasouri et al., 2004*).
- Phosphate buffers tend to precipitate polyvalent cations (e.g. Ca^{2+}), while also acting as a metabolite or inhibitor in various systems. Although phosphate-buffered saline (PBS) has an appropriate pKa, elevated levels of phosphate can damage cellular function, compromise gamete and embryo metabolic activity (perhaps via the Crabtree effect), disrupt organelle distribution, and interfere with intracellular ionic homeostasis, including pHi.

The simultaneous use of multiple different buffers allows for adjustment of pKa values to the desired range, while also permitting a reduction in individual buffer concentrations and potentially alleviating concerns for toxicity. Recent attention has focused on use of combination buffers in handling media for IVF to further refine the current mono-buffered systems (*Swain and Pool, 2009*).

Culture media – general composition and protein supplementation

Little has changed since the statement 'It remains true in 2011 that there is no culture medium available that is truly optimized for human embryo development' (*Harper et al., 2012*). Consequently, attempting to define consensus is limited by our relative uncertainty of what is optimal for human embryo development.

There are only a few concepts that have reached consensus status for medium composition. Because most culture media contain over 40 components, at different concentrations and ratios relative to each other, the number of possible combinations is limitless. As osmolality and pH are reviewed elsewhere in this document, the discussion here will be limited to the presence, form and concentration of nutrients. The following areas of general consensus are based on years, often decades, of evidence supporting their central role in metabolism and development of preimplantation mammalian embryos.

Antibiotics, mostly in the form of gentamicin, are a ubiquitous component of human embryo culture media. Although not required, the benefits outweigh the risks, making inclusion of antibiotics a standard practice in the industry (*Kastrop et al., 2007*; *Swain, 2015*).

Carbohydrate needs are met via pyruvate, lactate and glucose, with pre-compaction embryos preferring pyruvate followed by a shift to glucose for compaction and blastocyst formation (*Leese, 2012*). Most culture media provide pyruvate at concentrations between 0.2 and 0.5 mmol/l (*Morbeck et al., 2014a*, *2017*), reflecting both the concentration as measured in fallopian tubes (*Dickens et al., 1995*; *Gardner et al., 1996*; *Lopata et al., 1976*; *Tay et al., 1997*), and the concentrations derived via simplex optimization (*Lawitts and Biggers, 1991*). Results from the latter study suggest that pyruvate concentrations higher than 0.5 mmol/l may be detrimental. Perhaps not surprising, all but one now-discontinued medium provided pyruvate at ≤0.5 mmol/l (*Morbeck et al., 2017*).

Glucose, while not required for pre-compaction embryo development, and at one point considered detrimental to early cleavage divisions (*Quinn et al., 1995*), is now included as a standard component of all complex culture media on the market. Amino acids and EDTA are important contributors to the metabolic environment that limits the utilization of glucose during the pre-compaction stage (*Biggers and McGinnis, 2001*; *Houghton, 2012*; *Lane and Gardner, 1997*). Post-compaction, glucose becomes the preferred energy source and is supplied at ostensibly physiological levels of ~3 mmol/l in blastocyst-stage media (*Gardner et al., 1996*; *Morbeck et al., 2014a*) versus 0.2–1.0 mmol/l for 'single-step' or cleavage-stage media (*Morbeck et al., 2017*). The lack of consensus on glucose concentration probably reflects the embryo's ability to obtain its energy and carbon from diverse sources, including pyruvate, lactate and amino acids. It is worth noting that EDTA, an additive that was believed to be necessary to chelate potentially toxic metals, is used in all media at varying concentrations as it may be involved with glucose metabolism, limiting the availability of magnesium as a cofactor for glucose metabolism (*Lane and Gardner, 2001*).

Amino acids are ubiquitously present in culture media and in at least one protein supplement (*Morbeck et al., 2014a*, *2014b*, *2017*). Early iterations of culture media for human embryos were mostly amino acid free, e.g. Earle's medium (*Cohen et al., 1985b*) and Quinn's HTF (*Quinn et al., 1985*), although Ham's F-10 (*Leung et al., 1984*) and Menezo's B2 and B3 media (*Menezo et al., 1984*) contained amino acids based on Eagle's formulation (*Eagle, 1959*). While all amino acids were included in Gardner's blastocyst-stage media, only non-essential amino acids were included in cleavage-stage sequential media (*Gardner and Lane, 1998*) due to the possibility of reduced development of cleavage-stage embryos exposed to essential amino acids – at least when used at MEM (minimal essential medium) levels (*Lane and Gardner, 1997*). In contrast, the single-step media include most or all of the 20 amino acids (*Morbeck et al., 2017*). Amino acids play numerous roles in cellular function, including but not limited to acting as metabolites, antioxidants, osmolytes and buffers. Thus, there is a consensus that complex media should contain a robust complement of essential and non-essential amino acids. The selective efficiency of non-essential and essential amino acids in early stages needs further clinical research, but there seems to be consensus that both groups are needed for post-compaction development.

L-glutamine, an amino acid with many roles during embryo growth and thus considered an essential component of culture medium, breaks down spontaneously with ammonium as a by-product (*Kleijkers et al., 2016a*). There are concerns regarding the relative amount and impact of ammonium accumulation in culture media (*Biggers et al., 2004*), but the effect is magnified in 20% O_2 (*Wale and Gardner, 2016*). A simple solution has been the introduction of glutamine in a stable dipeptide form (*Lane and Gardner, 2003*). The use of dipeptide glutamine is now standard practice for all versions of complex ART culture media.

Protein is an important component of culture medium that is provided in several forms and, by consensus, should be included in all embryo culture media. Albumin is the most abundant protein in the oviduct (*Leese, 1988*) and thus the most common protein used in culture media. Albumin serves many functions, most notably as an antioxidant (*Bavister, 1995*; *Morbeck, 2015*). Lot-to-lot variations exist with human serum

albumin (HSA) (*Swain, 2015*), particularly with respect to the stabilizer octanoic acid (*Fredrickson et al., 2015*; *Leonard et al., 2013*). Human albumin is also available in a recombinant form (recHSA; *Bungum et al., 2002*), although exclusivity rights restrict its availability, mostly as a supplement for end-users to add to non-supplemented media. Even the sole supplier does not offer media pre-supplemented with recHSA, probably due to issues with cost of the product. So, while recHSA might offer theoretical advantages over HSA in terms of safety and product consistency, its routine use is unlikely while commercial exclusivity remains in place.

Complex protein supplements are common and have a strong market presence, particularly in the Americas. There are two classes of complex protein supplements: HSA solutions containing alpha and beta globulins or dextran. The latter is a surfactant and volume stabilizer, although there is little evidence for the benefit of using a polysaccharide along with albumin (*Campbell et al., 2013*). In contrast to dextran, alpha and beta globulins carry potential benefits that can yield improved live birth rates (*Meintjes et al., 2009b*). There are two formulations of protein supplements containing globulins: one where USP (US Pharmacopeial Convention) grade alpha and beta globulins are mixed with HSA and one that is a less pure fraction of human serum that contains both albumin and alpha and beta globulins. Both empirical observations and regulatory barriers limit the widespread use of these complex protein supplements globally. Globulin-containing products are poorly defined, can contain high levels of pro-oxidant metals (*Morbeck et al., 2014b*), and suffer from significant lot-to-lot variation (*Meintjes, 2012*). For these reasons, and with limited evidence for benefit, the proposed consensus is that industry should move to protein supplements that are the most defined, and, at some point, not a human product. The proposed consensus is that recHSA is theoretically safer and more consistent than HSA, which is safer and more consistent than globulin-containing formulations.

Human serum, the predominant form of protein supplement used during the early years of clinical IVF (*Edwards and Steptoe, 1983*), is inherently variable and can carry risks to the health of the offspring. Thus, the field reached consensus in the 1990s that serum was no longer suitable as a supplement for human embryo culture.

Phenol red is included in many culture media as a QC measure to monitor medium pH, rather than it having any role in embryo development (*Swain, 2015*). However, phenol red addition carries some hypothetical risks, based on its oestrogenic activity (*Moreno-Cuevas and Sirbasku, 2000*) or as a source of ROS (*Nakayama et al., 1994*), thus making it a component for which risks outweigh benefits. In this era of microdrop culture, where volumes of media are <100 µl, a colour indicator is neither necessary nor beneficial because visual assessment of colour changes in such small volumes is very hard to standardize.

However, in other areas, consensus is lacking, as described below.

Lactate concentrations vary widely. An important partner with pyruvate during the first few days of embryo development, lactate is intimately involved in pyruvate uptake and metabolism (*Lane and Gardner, 2000b*) and production of NADH and thus REDOX potential in blastomeres (*Dumollard et al., 2007*, *2008*). Nearly all studies of reproductive fluids report lactate concentrations of 5–10 mmol/l (*Dickens et al., 1995*; *Gardner et al., 1996*; *Lopata et al., 1976*; *Tay et al., 1997*) and the concentration chosen, at least for single-step media, is within this range to give a lactate:pyruvate ratio of approximately 20 (*Morbeck et al., 2017*). Lactate concentrations at 2 mmol/l, which result in lactate to pyruvate ratios of 2 to 5, have been introduced by some designers (*Morbeck et al., 2014a*), although these low lactate concentrations and lactate:pyruvate ratios have not been rigorously studied in animal models and thus lack consensus.

Calcium and magnesium, in terms of their absolute and relative concentrations, also vary widely with a nearly 10-fold difference in magnesium concentrations (0.2–1.8 mmol/l) that result in a 10-fold difference in the ratio of calcium to magnesium (0.6–7.1; *Morbeck et al., 2017*). The significance of the difference in Ca:Mg ratios is unknown, although calcium signalling after fertilization is a likely target, and differences in this ratio could affect developmental potential (*Lu et al., 2018*). Thus, absolute concentrations of Ca^{2+} and Mg^{2+} and their ratio lacks consensus.

Hyaluronan, a glycosaminoglycan, and taurine/hypotaurine, an aminosulfonic acid, are added to some but not all culture media. Benefits of hyaluronan have been described for embryo transfer media (*Bontekoe et al., 2010*), but there is a limited clinical evidence base for benefits in embryo culture (*Gardner et al., 1999*). Recent work in bovines and sheep, particularly after cryopreservation, has shown outcome improvement (*Dattena et al., 2007*; *Palasz et al., 2008*). In contrast, taurine is used in culture media for domestic animals with proposed antioxidant and osmolytic benefits (*Devreker and Hardy, 1997*; *Dumoulin et al., 1997*).

There are numerous micronutrients present in some culture media, components with minimal evidence basis and thus lack of consensus among media manufacturers. These factors include antioxidants, such as lipoic acid, essential nutrients like choline, and vitamins, including but not limited to the B vitamins, vitamin C and vitamin E.

Insulin has been included in a minority of embryo culture media, possibly as a reflection of the common inclusion of insulin, transferrin and selenium in cell culture media (*Shamsuddin et al., 1994*). A recent study showed that insulin added to a single-step human embryo culture medium improved embryo development and ongoing pregnancy rates (*Fawzy et al., 2017c*), although further studies are warranted to validate these findings.

Growth factor or cytokine supplementation of commercial human embryo culture media is currently limited to granulocyte-macrophage colony-stimulating factor (GM-CSF), a multifunctional cytokine present within the oviduct and uterine epithelium during the peri-implantation period (*Robertson, 2011*). A large RCT showed a benefit of GM-CSF in select patient populations (*Ziebe et al., 2013*), but only when the level of HSA was reduced. In the control group the lower level of HSA (2 mg/ml) had lower outcomes than the standard 5 mg/ml. Similar to recHSA, manufacturer exclusivity limits the availability of recGM-CSF to one culture medium. This commercial exclusivity limits the broad

application and clinical assessment of GM-CSF, making it premature to establish consensus on its use.

In conclusion, reaching a consensus on the composition of human embryo culture medium is difficult due to the fact that individual components have not – and cannot – be optimized in patients, notwithstanding the possibility that one culture medium might not be suitable for all patients. Hence the conclusions summarized here are actually an expert opinion based on industry practices. There are several components that can be considered standardized and several others that merit consideration for consensus. These two groups of components comprise a small minority of the total constituents of culture media on the market, making most of the composition not standard.

- Carbohydrates – pyruvate, lactate and glucose – are universally added to culture media, as are most if not all common amino acids.
- Glucose and pyruvate concentrations fall within a relatively narrow range.
- Amino acid concentrations vary considerably; glutamine is notable given its lability and hence its routine inclusion as a stable dipeptide.
- Inclusion of the antibiotic gentamicin is standard.
- Serum-free culture is standard.
- Protein supplementation has considerable variation and presents an opportunity for standardization. Complex protein supplements may bring benefits, yet are poorly defined and variable from lot to lot. Moving to the most defined protein available, and preferably not from human sources, is warranted. Ideally, protein is added to the culture medium by the manufacturer.
- Inclusion of phenol red remains variable and is generally unnecessary in media used for extended culture.

In practice the ART field is faced with the challenge of improving the composition of culture media while acknowledging that there is no suitable model in which to prove the principles first. Harper and colleagues, when providing recommendations for how to bring new culture media to market, stated: 'If we aim to change the design of culture media in significant ways, we must carefully assess the risk of those changes, as well as the potential benefits.' Yet new single-step media with significant changes in design were recently introduced with no published animal studies and little clinical oversight (*Hardarson et al., 2015*; *Sfontouris et al., 2017*). The composition of new culture media, such as a new single-step medium with low lactate, are shrouded in mystery, leaving practitioners, and thus patients, with little evidence or assurance of safety (*Sunde et al., 2016*). An area where we most lack consensus is how to continue to make improvements to culture media in a safe yet effective manner.

Culture media – sequential or 'single-step' media for human embryo culture

Two different approaches to developing embryo culture media have been proposed, more than a century after the first cell tissue culture system (simple salt solution) was described by Ringer and Ludwig. Aiming to mimic the reproductive environment (the so-called 'back to nature' principle), experimental embryologists in the 1970s tailored culture medium composition to closely match the chemistry of the female reproductive tract. An example of the back to nature principle is the sequential embryo culture system. The second group of media was designed to optimize growth *in vitro*, having embryo development as an endpoint to optimize composition, to some extent ignoring existing formulations and principles. These 'simplex optimization', or SOM, media were described by scientists supported by the National Institutes of Health in the USA during the 1990s, in a team effort that was colloquially referred to as 'The Culture Club'. To formulate SOM media the performance of each ingredient is evaluated separately using the 'simplex optimization' process – a mathematical system developed first for the mouse in the laboratory of John Biggers at Harvard University 25 years ago. The advantage of this approach over the sequential system is that there is no consideration of fallopian and uterine conditions, and that embryos can be left alone without altering conditions for 5 days or longer. The core question is whether this perceived advantage comes at a cost.

Gardner and Lane (1997) were the first to suggest that 'in order to optimize mammalian embryo development in culture, sequential media are required, each designed to meet the changing requirements of the developing embryo'. The need for sequential changes in their culture media was a logical conclusion of their experiments. This compelling principle and the 'back to nature' approach in general have been challenged in part because the approach is reliant on the analysis of oviductal and uterine components, and such analyses may have shortcomings (*Biggers, 2002*; *Cohen and Rieger, 2012*).

The suggestion that either the single-step SOM approach or sequential culture is better is not based on scientific arguments and cannot be decided after evaluation of the literature. More than a thousand papers and abstracts have been written about culture media systems and a considerable number of thorough reviews have been published comparing the two approaches (*Dieamant et al., 2017*; *Sfontouris et al., 2016*; *Youssef et al., 2015*). Analyses like this are unfortunately compromised by the quality of the original comparative studies and a multitude of confounders affecting laboratory results. Common confounders such as support media for gamete handling, protein supplementation and details of incubator QC aspects are often ignored by authors, and are hence unavailable to reviewers.

The recent systematic reviews plead for additional studies (including repeat trials), larger investigations and improved quality of evidence. There are five important aspects known to affect the reliability of RCT: (i) appropriate randomization; (ii) allocation concealment; (iii) blinding; (iv) limiting technological bias (such as rigorously applying manufacturer's instructions and reducing the effect of known confounders); and (v) independent statistical evaluation. Trials evaluating the two culture systems are often lacking in most of these areas. The debate over evidence-based practice has been long and tedious in reproductive medicine and particularly the use of the RCT as the sole argument to prove or disprove a hypothesis has been questioned (*Cohen and Alikani, 2013*; *Harper et al., 2012*).

Harper and coworkers (2012) suggested not just paying attention to RCT studies and the typical evidence base when evaluating new and existing clinical embryology technologies. This model of medicine and evidence by itself was not new, but the context was refreshing. They considered seven premises: (i) Is

the technology based on an appropriate hypothesis? (ii) Were studies in animals performed? (iii) Were spare gametes and embryos evaluated? (iv) Was the technology validated in small series studies? (v) Were RCT performed? (vi) Is the technology relevant from a routine application point of view? (vii) Is the technology economically sensible?

It is suggested that the 'Harper model' be considered seriously, as there is sufficient merit to each of the 'common sense' premises. If each technology is evaluated for these seven themes, and one aspect is not considered more important that any of the others, the lack of RCT might be considered acceptable (in some cases), and technologies can be evaluated (and compared) according to their Harper model score. Using this model, a score can be derived within a few years after the introduction of the technology, rather than the evidence-based approach, which can drag out over decades, often causing considerable debate and confusion. This was illustrated by the work of David Gardner and colleagues describing the first sequential culture medium system G1 and G2, nearly 20 years ago (reviewed in *Niederberger et al., 2018*). Those studies fulfilled the Harper model criteria in short order, although later RCT comparing sequential media against single-step KSOM-derived culture in systematic reviews did not show differences (*Dieamant et al., 2017*; *Sfontouris et al., 2016*).

Use and management – cold chain and storage

Manufacturers provide specific guidelines regarding transport and storage of human IVF culture media products, with little real difference between suppliers. Guidelines for storage and handling are based on manufacturers' experience, professional organization directives, and an example set by the associated field of cell tissue culture. General recommendations are:

- Culture media products must be stored at refrigerated temperatures according to the manufacturer's instructions (2–8°C) and kept away from light, particularly sunlight.
- Storage conditions and expiry date should be clearly shown on labels and product inserts.
- Products should be used in order of batch or lot numbers.
- Products should never be used after expiration of the shelf life indicated on the product or inserts.
- Bottles of ready-to-use culture medium should not be opened once, closed and reopened again for use later.

Culture media effects for ART do not just start during egg retrieval, but are a function of transportation as culture media may go out of their specified tolerance range during transport and storage. Culture medium is produced at room temperature and may be kept for a while at ambient environments before final packaging and refrigerated storage. It is obviously safe to assume that culture medium tolerates some changes in temperature, but potential detrimental effects, if any, are a function of exposure changes, duration and frequency of temperature fluctuations. There are two extreme events that may occur in cold chain failure: freezing or becoming overheated.

Effect of freezing

When the ambient temperature drops below 0°C it may take a while before a medium freezes, due to the shipping box and its thermal mass, which reduce the impact of temperature excursions. The general composition of culture media is such that they will not freeze immediately but will first super-cool, without inducing ice crystallization. Recommended storage conditions are +2°C to +8°C, indicating that manufacturers rely on a safety margin. Once a culture medium freezes, it will have to be discarded because the freezing can cause the liquid part to become non-homogeneous and subsequently the medium might not reconstitute properly. If the culture medium is protein supplemented, there is an increased risk of denaturation, as the liquid portion might have a sub-optimal osmolality and pH. Low temperatures do not have much effect on mineral oil.

Effect of elevated temperature

The cold chain might be broken due to transportation delays or temporary storage at elevated temperatures. A slightly elevated temperature between refrigeration and room temperature for up to a week might not be detrimental. The main effect of elevated temperature exposure is oxidation and a reduction in the concentration of active components. Through deamination amino acids and proteins might slowly release ammonium ions that have been shown to have a negative impact on the embryo; an effect that is worse when the monopeptide version of glutamine is used (as opposed to the more stable alanyl-glutamine or glycyl-glutamine dipeptide forms) or in protein-supplemented culture media. The process is slow, but effects may be noticeable after several days, and occurs even when samples are held at stable refrigerator temperatures: protein-free culture media do not have any marked ammonium ion build-up *in vitro* at 37°C or during 6 weeks in the refrigerator, but undiluted protein supplements showed considerable deamination after just a few days at 37°C (*Kleijkers et al., 2016a*). The actual levels of ammonium ions and specific effects at very low concentrations are unknown and effects could be brand-dependent. The reactions are O_2- and temperature-dependent and degradation can be calculated. A similar problem can occur in the event of a laboratory refrigerator malfunctioning.

Logging thermometers are sometimes sent in shipments. From their data, the mean kinetic temperature (MKT) can be calculated, a measure routinely used in the pharmacological industry as a simplified way of expressing the overall effect of temperature fluctuations during storage or transit of perishable goods.

There are no known international recommendations for storing IVF culture media. Regular residential refrigerators and freezers, which have very poor temperature stability, are still seen in IVF laboratories. Appropriate refrigeration can only be achieved with clinical grade ('pharmacy') refrigerators, which generally reduce fluctuations and failures. These units have wire shelves and compartments for improved circulation, and the doors do not have any shelves for storing samples. Combined side-by-side refrigerator/freezer units should not be used. Storage efficiency is dependent on load, so that empty and completely full refrigerators under-perform. A built-in unit should have a fan in the front.

Monitoring refrigerators and freezers using an external/secondary method is highly advisable and typically required by accreditation bodies. Monitoring should be periodic (at least daily). The Centers for Disease Control and Prevention (CDC) recommends NIST-calibrated certified thermometers using a glycol-filled bottle in addition to

display temperature. Dataloggers may be helpful. An audible alert is useful, as are self-closing doors, open door alerts and keyed locks.

Effect of light

Visible light is non-existent in the reproductive tract, but during laboratory exposure both gametes and embryos, as well as the products used for embryo culture, are exposed to visible light of various intensities (review by *Pomeroy and Reed, 2013*). The sources of light in ART laboratories are ceiling lights and microscopes (*Pomeroy and Reed, 2013*) and *Ottosen et al. (2007)* have shown that illuminance (lux) and irradiance are greater from microscopes than ambient light by a factor of 10–20 × . Harmful light-mediated effects have been described in invertebrates, hamsters, mice and cattle, but the direct effect of light on human embryos is basically unknown (*Ottosen et al., 2007*). Blue and ultraviolet light are found to be the most harmful and the effects can be suppressed by use of red and green filters on microscopes. Important indirect peroxidation effects of light on mineral and silicone oil have been documented (*Otsuki et al., 2007*). Indirect effects of light via hydrogen peroxide have also been found in HEPES-buffered media and culture media including essential amino acids like tryptophan and tyrosine. The intensity of the exposure (irradiance) is an important aspect to consider, as well as the duration of exposure and actual wavelength. Photo-oxidation (also known as peroxidation) of oil can occur when exposed to ultraviolet. Other compounds affected by light exposure are hydrocarbons, nitrogen and sulphur.

Test equipment – calibration and certification

Laboratory environment

VOC and air contaminant monitoring: Equipment with the proper sensitivity must be used (*Hall et al., 1998*). Meaningful VOC values are measured as ppb and not ppm. Sensitized photo-ionization detection (PID) devices are used for screening with sensitivities not lower than 100 ppb. Frequent calibration (at least semi-annually is recommended) is typically performed at the factory with a reference gas such as isobutylene. Heated metal oxide sensors (HMOS) are not recommended as their sensitivity is not acceptable. For the continuous measuring of ppb, a photoelectric absorptiometric principle is used, applying a sensor cartridge (150–1000 tests).

Particulates: Particle size to be measured should be defined. Most particle counters are certified and calibrated by the manufacturer using NIST-traceable particles; typically, in-house calibrations are not required. For ISO-certified clean rooms, particle counts should be performed according to *ISO 14644*, although a recent consensus on IVF laboratory air quality recommends a more practical approach (*Mortimer et al., 2018*).

Temperature: Temperature measurements should be tailored to the purpose and acceptable tolerance for the equipment being tested. Temperature measuring devices for working surfaces can be accepted with an accuracy of ±0.05°C. Thermometers should be calibrated at regular intervals, at least annually to conform to *ISO 15189:2012*.

Humidity: Control of room humidity is strongly recommended to minimize the risk of fungal growth and contamination, for staff comfort, and to minimize evaporation during the open handling (no oil overlay) of gametes and embryos. Inexpensive measurement devices can be used successfully, but typically last only for months. Continuous and/or wireless humidity sensors are calibrated at installation and should be recalibrated at least twice per year.

Culture environment

Temperature: When measuring the temperatures of incubators or other highly sensitive environments, a tolerance limit of no more than ±0.1°C is recommended. Thermometers should be calibrated against a NIST-traceable reference device at least twice per year.

pH: Monitoring of pH is commonly used as a means of validating culture medium performance (*Swain, 2012b*) as described above. However, with the consistency of modern culture media, the stabilizing effect of bicarbonate buffering, compromised measuring resolution and inter-measurement variation, routine single pH measurements may be of questionable value. Hand-held pH meters need to be calibrated before each use. Furthermore, one must ensure that the pH probe has a meaningful resolution and range for IVF applications, is designed for IVF micro-volumes, and that an immediate reading at 37°C can be obtained. Continuous measurement of pH *in situ* (inside the incubator) is possible using continuous sensors with certification and calibrations determined by the manufacturer. One example of continuous pH monitoring is SAFE Sens® (Blood Cell Storage Inc., Seattle, WA, USA), which employs an optical fluorescent measurement technology. Each disposable sensor contains a proprietary pH-sensitive fluorescent dye; the fluorescent signal is read continually at a set frequency and converted to a pH value. Each lot of sterile sensors lasts 7 days and is certified by the manufacturer.

Blood-gas analyzers: These instruments provide instant and accurate pH readings without having to rely on inaccurate hand-held pH meters or more expensive continuous monitoring devices (*Swain, 2013*). In addition, blood-gas analyzers provide helpful pCO_2, pO_2 and electrolyte information that can aid in the overall quality management of the culture system. The hospital-type analyzers are expensive and require daily maintenance and calibration. Newer handheld devices allow for pH, pCO_2, pO_2, and electrolyte readings, using disposable cartridges with integrated calibrators.

Oxygen: O_2 can be measured reliably with mechanical devices, electronic measuring devices, or the pO_2 can be determined with a blood gas analyser. Electronic hand-held devices should be calibrated regularly (daily, monthly, periodically) as specified by the manufacturer. A calibration gas mixture is frequently required for proper calibration. In a growing number of countries, the classic Fyrite® fluid for measuring O_2 or CO_2 requires hazardous goods shipment or is not even available.

Amperometric (galvanized or voltage-sensitive) and optical sensors are used in these applications, with an optical sensor requiring the least maintenance. Over time, both types of O_2 sensors can suffer from ageing effects leading to measurement drifts. A reference electrode (amphoteric) or reference LED (optical) or backup control unit is always advisable.

Laboratory equipment and real-time monitoring

Real-time monitoring (RTM) has long been considered impractical because of

the lack of accurate CO_2 sensors, the difficulty of connecting a large number of sensors, and the cost of cabling and adding sensors and data transmitters. Today, with the universal availability of low-cost wireless technology, internet, smart phones and tablets this is no longer the case, and there are now affordable solutions that provide vital information in real time. RTM systems can reduce 'loss' by equipment failure and thus provide the Embryology Laboratory Manager with increased safety and reliability. Also, regulators see the benefits of monitoring and this requirement is now integrated in professional guidelines (*Magli et al., 2008*), regulatory requirements (*Commission Directive 2006/86/EC*) and accreditation standards (*ISO 15189:2012*).

It is possible to connect analogue sensors for temperature and gas levels (CO_2, O_2 and VOC). If feasible, sensors independent from the equipment being monitored should be used. This makes it possible to detect equipment sensor drift and allows verification of a manufacturer's performance claims and may detect environmental factors such as electrical failure.

Air pressure, relative humidity, air flow sensors and particle counters can be connected to an RTM system but these parameters are usually integrated into a building management or monitoring system (BMS). Laboratory ambient air monitoring should be part of the laboratory's RTM system because deviations in ambient temperature have consequences on the temperature regulation of microscope heated stages.

Digital signals that can be monitored in real time are door status, equipment alarm signals. It is even possible to read digital RS232 or RS485 interfaces.

Modern web-based systems provide accurate and effective control of equipment, the data are accessible remotely over a secure internet connection and intelligent alarms warn the Laboratory Manager in case of an unexpected event or equipment malfunction or failure. To increase reliability, technical alarms (sensor break or monitoring equipment failure, network failure) should be possible and this aspect should be taken into account when a monitoring system is chosen. With modern technology it is possible to send alarm notifications by telephone, email or SMS, but the alarm messaging program should be bidirectional so that alarm acknowledgement is possible (and logged). In case of no reaction within a predefined timeframe, an automatic cascading system should be activated.

Blood gas analysers provide instant and accurate pH readings without having to rely on inaccurate hand-held pH meters or more expensive continuous monitoring devices (*Swain, 2013*). In addition, blood gas analysers provide helpful pCO_2, pO_2 and electrolyte information that can aid in the overall quality management of the culture system. The hospital-type analysers are expensive and require daily maintenance and calibration. Newer hand-held devices allow for pH, pCO_2, pO_2 and electrolyte readings, using disposable cartridges with integrated calibrators.

Validation: In *ISO 15189:2012*, the international standard for accreditation of medical laboratories, validation is defined as 'confirmation, through the provision of objective evidence, that the requirements for a specific intended use or application have been fulfilled'. IVF is a process, and a basic objective of validation is to ensure that each step and each variable of the process is identified and controlled, and process variability is reduced so that the finished product meets customer requirements (consistent high pregnancy rates). Certainly, in IVF the quality of the 'end product' cannot itself be measured, so each contributing factor (infrastructure, equipment, utilities) and all the steps of the process need to be known and controlled.

Validation should be performed for new premises, laboratory equipment, utilities and processes and procedures and should result in written reports. During the validation, in-process controls should be defined to monitor the process.

Equipment needs to be validated to provide a high degree of assurance that it will consistently meet its predetermined specifications with minimal variation. Equipment validation is broken down into three phases: 'Installation Qualification' (IQ), 'Operational Qualification' (OQ), and 'Performance Qualification' (PQ). As the first step, IQ ensures that the equipment is correctly installed according to the manufacturer's specifications, e.g. a new incubator needs be installed on a solid, vibration-free surface, the room temperature should be within a defined range, the instrument should be connected to CO_2 and mains power. During the next step, OQ, the equipment is calibrated and tests are performed to document a baseline of the critical parameters of the equipment. For the incubator, this is defining set-points for CO_2, temperature, O_2 and a verification of these parameters with independent, calibrated measuring equipment. The PQ phase then tests the ability of the incubator to perform over long periods within an acceptable tolerance range.

The equipment, utility and system should then be maintained, monitored and calibrated according to a regular schedule by responsible personnel with appropriate qualifications and training. Parameters of calibration and equipment verification should be traceable to international standards. Calibrated equipment should be labelled, coded or otherwise identified so that the calibration status and recalibration due date are clear. If equipment is not used for a certain period of time then the calibration status needs to be verified before use.

Consensus guidelines

During the consensus meeting, the attendees formed statements based on the best available evidence and, if lacking, their expert opinion. In our deliberations it was not possible to conclude that there were certain aspects which were more important than others. Rather we agreed on an important fact first formulated by Don Rieger, that everything is important in the IVF laboratory. In TABLE 2 we summarize some of the points we focused on. General recommendations were aimed at creating a scenario whereby the laboratory or culture of eggs and embryos is more a case of limiting the cumulation of stresses. It is accepted that the whole process may not be optimal, however, the fewer the stresses the higher the chance of normal offspring. In many discussions, it was noted that high-level evidence was lacking. It was also a general observation that properly designed, conducted and reported RCT were lacking. This is unfortunate, especially because randomizing what we do in the IVF laboratory should not be considered a 'scientific experiment', but a mere evaluation of what we do

TABLE 2 ALTHOUGH EVERYTHING IS IMPORTANT IN THE IVF CULTURE SYSTEM, A LIST OF 20 SUGGESTIONS IS PRESENTED HERE; THESE ARE NOT PRESENTED IN ORDER OF IMPORTANCE, NOR ARE THESE THE ONLY POINTS COVERED IN THE CONSENSUS DOCUMENT

IVF culture aspect	Consensus suggestion
1	The chance of every patient having a healthy baby is the most important outcome. Cumulative live birth rate per woman or started cycle and other parameters should be monitored. High-level evidence is still lacking.
2	Gamete and embryo assessment practice should be based on expert or professional body, peer-reviewed, published and evidence-based guidelines.
3	Physicochemical conditions must be maintained during assessment, but evidence for optimal timing and frequency is lacking.
4	Evaluation of embryo culture must include embryo viability (in-vivo development) after transfer. Appropriate measures are implantation rate, live babies born per embryo transferred, embryo and fetal loss rate.
5	Temperature validation is critical for each step of IVF. Evidence supports maintenance of 37°C during all aspects of culture, but humidified and non-humidified incubator environments may both be supportive depending on conditions.
6	Measurement of pH and of CO_2 concentration can provide effective QC. Evidence supports culture of embryos in a low oxygen (normally 5% O_2) iso-atmospheric condition.
7	Micromanipulation validation should include temperature mapping of the stage (dish) to determine the maximum period the oocyte can be maintained at ~37°C and the lowest light setting should be chosen.
8	It is critical that mechanical (shear) stresses, as well as temperature changes and pH shifts, be minimized during all assessment and pipetting procedures.
9	Validated QM (SOP) programmes for routine monitoring of incubator performance should include daily assessment of temperature, CO_2, O_2 and humidity. Pre-established targets with tolerances (warning and control limits) for each variable are required as benchmarks.
10	Incubator supply gases should be filtered to remove particulates and contaminants. For pre-mixed incubator supply gases, the individual gas levels in the mixture should be verified and liquefied CO_2 level must be monitored.
11	Select buffers (i.e. HEPES and MOPS) appear to be safe for stabilizing pHe outside the culture incubator, but consequences of prolonged exposure are unknown.
12	Commercially manufactured culture medium intended for human ART should be used with adherence to protein supplementation guidelines from manufacturers.
13	There is currently insufficient evidence to support the addition of bioactive compounds, such as growth factors, to gamete and embryo culture medium. Further safety and efficacy studies are required before their routine inclusion.
14	SOP to verify acceptability for receipt and use of all contact materials, in accordance with best practice and local regulations. This includes keeping permanent records of Certificates of Analysis, lot/batch numbers. The laboratory should monitor ongoing performance of these materials.
15	Single-step media and sequential culture are both supportive of development and high outcomes. Current evidence is limited, and insufficient to demonstrate one culture system to be superior over the other.
16	Culture media must be kept and monitored under manufacturer's recommended storage conditions. Appropriately maintained and manufactured storage refrigerators must be monitored diligently.
17	Independent continuous monitoring of critical parameters of laboratory infrastructure and equipment is desirable and may be mandatory in certain countries.
18	Culture media batches can be validated by using accepted KPIs such as the Vienna consensus.[a]
19	Spermatozoa for ART must be efficiently separated from the seminal plasma environment as soon as possible after ejaculation.
20	Spermatozoa preparation for ICSI should be held at ambient temperature to minimize ROS generation.

ART = assisted reproductive technology; ICSI = intracytoplasmic sperm injection; KPI = key performance indicator; QC = quality control; QM = quality management; ROS = reactive oxygen species; SOP = standard operating procedure.

[a] *ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017*.

every day. When discussing what is best to do in the IVF laboratory, the group considered cumulative live birth rate to be the outcome of interest (*Maheshwari et al., 2015*; *Wilkinson et al., 2016*). Ultimately not only the cumulative live birth rate, but also data on safety (for both the woman and the forthcoming child), time to pregnancy, and costs need to be taken into account. For many of the topics discussed, high-level evidence on outcomes was lacking. Also, the group primarily relied on data reported per woman or per started cycle, rather than per embryo or per transfer, as the latter could be misleading when evaluating any part of the IVF treatment (*Griesinger, 2016*). Examples mentioned during the meeting were reports on increased fertilization rates, and increased implantation rates, while in these same studies a decrease of live birth rate was reported (*Kleijkers et al., 2016b*; *Mastenbroek et al., 2007*). The group acknowledged the shortcomings of these outcome measures in evaluation of laboratory treatment efficacy, while at the same time recognizing their value for validation and monitoring of laboratory performance (*ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017*). Validation and monitoring are used to ensure laboratory performance within predefined limits, but the decision on what to do comes down to treatment efficacy.

Assessment practices

It is critical to select and follow evidence-based guidelines for good laboratory and assessment practice. Gamete and

embryo assessment practice should be based on expert or professional body, peer-reviewed, published, evidence-based guidelines that are the most appropriate for each setting.

All possible measures should be taken to maintain physcochemical conditions during assessment (considering assessment timing and frequency, dish set-up/oil overlay, buffering, temperature control, consumables, equipment and laboratory environment).

It was agreed that at present there is no evidence for the optimal timing and frequency of assessments. Until RCT data are available, these practices should be established for each individual laboratory considering risks and benefits. In doing so, each laboratory should define, monitor and validate that their assessment practice is effective and fit for purpose within their operating environment, using published KPI.

Ultimately, the evaluation of embryo culture must include embryo viability (in-vivo development) after transfer. Appropriate measures of in-vivo development include implantation rate, live babies born per embryo transferred, embryo loss rate and fetal loss rate. Conversely, pregnancy rate, clinical pregnancy rate, live birth rate, miscarriage rate and any cumulative rate do not account for the number of embryos transferred and/or the number of transfers, and thus are not appropriate measures of embryo viability.

Temperature

Available evidence supports the maintenance of human oocytes and embryos at 37°C during culture. It could be that there is a range of acceptable temperatures, but this range has not yet been determined. If the available equipment cannot provide a tight temperature control, then a slightly lower temperature is probably better than a slightly higher one.

Humidity

Humidified and non-humidified incubators can both yield acceptable clinical results, but currently available comparisons in humans seem to demonstrate a benefit of humidified culture (*Fawzy et al., 2017a*); specific culture conditions, such as oil volume/depth, medium surface area to oil interface, ratio of oil to medium, room humidity, pre-humidification of oil, medium droplet size, as well as medium changes, can affect individual results (*Mortimer et al., 2018*). As part of the validation of their systems, individual laboratories are encouraged to determine whether humidity is required in their culture system, while respecting local legislation and regulations.

Further research is encouraged to identify the critical parameters in defining these systems.

CO_2 and medium pH

Technically, it is the partial pressure of CO_2, rather than its proportion of the gas mixture, that maintains the pH of a bicarbonate-buffered solution. Therefore, if reporting % CO_2 it should be clarified whether this measurement is at sea level, as the relative proportion is affected by altitude.

Medium pH is a critical factor in embryo culture. Because pH is dependent upon the CO_2 concentration in a bicarbonate-buffered solution, then the measurement of pH and of CO_2 concentration can provide effective QC.

The decision about which of these parameters to measure and the frequency of measurement should be based on the laboratory's policies and SOP (with reference to established targets and tolerances). Measurements should be conducted using calibrated and validated sensors that are independent of the incubator (i.e. in addition to the incubator's displays).

Oxygen

It was the general consensus that there is robust evidence to support culture of embryos in a 5% O_2 atmosphere (although one expert noted that they felt the evidence is equivocal for Day 3 culture). There is evidence that there is greater efficiency in embryo culture with 5% O_2, but no studies have compared the health trajectories of children born from embryos cultured in 5% versus 20% O_2, and it was the consensus that these studies should be performed using retrospective data.

Micromanipulation

There are very few studies that address the optimization of sperm preparation methods for ICSI, although there is evidence that direct pelleting of spermatozoa from whole semen should be avoided if possible.

During the ICSI procedure, the aim is to maintain the temperature of each oocyte in the dish as close to 37°C as possible throughout the duration of the procedure, regardless of microscope stage temperature. This is best achieved with the use of a heated stage or warming plate. Validation of the micromanipulation rig should include temperature mapping of the stage and the dish to determine the maximum period that an oocyte can be maintained at 37°C in the injection area. Achieving this is only possible if the ambient environment is constant. If the external environmental conditions are changed, the system must be revalidated. For this reason, micromanipulation rigs should not be located in areas of active air flow.

Oocytes are sensitive to the blue-green end of the visible light spectrum, so these wavelengths should be avoided where possible. The ICSI procedure should be performed at the lowest intensity of light possible while still being able to visualize the gametes precisely.

It is critical that mechanical (shear) stresses be minimized. Preparation of oocytes for ICSI requires removal of cumulus oophorus and corona radiata cells. To minimize exposure to mechanical (shear) stress, complete removal of these cells is unnecessary beyond that required to perform the task effectively. In addition, the diameter of the stripping tip should be appropriate for the diameter of the particular oocytes.

Workstations

Workstations should be designed and operated to maintain oocytes and embryos as close to 37°C as possible while also minimizing pH shifts. When working with dishes without oil, every possible precaution must be taken to minimize evaporative cooling.

Travel distances between workstations and incubators should be as short as possible. This is an important matter of ergonomics in laboratory design.

Incubators

A validated QM programme for regular, routine monitoring of incubator performance should use independently calibrated devices for daily assessment of temperature and CO_2, with O_2 and humidity monitored as appropriate according to SOP.

Pre-established targets with tolerances (warning and control limits) for each variable are required as benchmarks for assessing performance. Data should be captured with dataloggers or hand-entered into control charts. Readings that cross warning or control limits should be investigated promptly.

The use of incubators that have thermal conductivity sensors for CO_2 measurement is discouraged. If incubators with thermal conductivity CO_2 sensors *are* used, humidity measurement and management are mandatory.

Incubator supply gases should be filtered before they enter the incubator, to remove particulates and gaseous contaminants. For pre-mixed incubator supply gases, the individual gas levels in the mixture should be verified. For incubators with internal gas mixers, there must be verification of the gas mixture achieved within the incubator.

CO_2 supply pressure must be monitored to ensure that there is always liquefied gas in the supply cylinder. This is critical to reduce the risk of release of VOC that are dissolved in the liquid CO_2 (*Mortimer et al., 2018*).

Handling practices

Every attempt should be made to maintain the temperature of the oocytes and embryos as close as possible to 37°C in every step of the IVF and in vitro culture process. This could be achieved through the use of a controlled environment chamber, but there are no RCT data available at this time to establish this benefit.

Care should be taken to manage heat loss in pipettes and oocyte collection needle sets. For example, holding multiple COC in the pipette while searching for other oocytes during the retrieval procedure should be avoided.

If a carrier is to be used when moving dishes between an incubator and a workstation, the choice of carrier type should be based on results from simulated procedures in which temperature of the dish contents was assessed.

The frequency of oocyte and embryo assessment has already been addressed in other consensus publications (*Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology, 2011*; *ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017*).

Culture media – buffering and pH

Maintaining a narrow and stable pHe is important for gamete and embryo quality. Ideal pHe is difficult to define and may vary based on media and ingredients (single versus sequential media; 7.20–7.35), as well as temperature. The CO_2 level appropriate for each laboratory's culture conditions must be determined and maintained closely. The appropriate CO_2 level will vary based on elevation and media composition.

Select buffers (i.e. HEPES and MOPS) appear to be safe for stabilizing pHe outside the culture incubator.

Culture media – general composition and protein supplementation

IVF laboratories should use products in general conformance to the manufacturer's instructions, and to local regulations.

Culture and micromanipulation media should contain protein, carbohydrates, a complex mixture of amino acids with glutamine in the form of a dipeptide, and usually antibiotic(s). The use of phenol red as a pH indicator is optional.

For reasons of quality, consistency and reproducibility, laboratories should use commercially-manufactured culture medium intended for human ART use. In certain jurisdictions this is a regulatory requirement. It should be noted that supplementation of media with exogenous protein beyond the manufacturer's recommendations could change the composition and performance of media.

A majority of the practising embryologists in the group expressed a belief that manufacturers should provide the formulations of culture media based on the need for a consensus regarding what constituent should be added to a culture medium and at what concentration; there were concerns that undeclared constituents of a culture medium can lead to unpredictable harm to future generations. Several members of the consensus, including Dr Don Rieger, abstained from this discussion due to potential conflicts and for other reasons.

It was the panel's opinion that there is currently insufficient evidence to support the addition of bioactive compounds, such as growth factors or complex proteins, to gamete and embryo culture medium, and that safety and efficacy studies are required before their routine inclusion.

IVF laboratories must have policies and SOP to verify acceptability for receipt and use of all contact materials, in accordance with best practice and local regulations. This includes keeping permanent records of certificates of analysis, and lot/batch numbers. The laboratory should monitor ongoing performance of these materials.

Culture media – sequential or 'single-step' media for embryo culture

A recent Cochrane Review (*Youssef et al., 2015*) concludes that there are no differences in outcomes between the two culture systems, and this conclusion is supported by other systematic reviews (*Dieamant et al., 2017*; *Sfontouris et al., 2016*). However, *Sfontouris et al. (2016)* concluded that: 'Although using a single medium for extended culture has some practical advantages and blastocyst formation rates appear to be higher, there is insufficient evidence to recommend either sequential or single-step media as being superior for the culture of embryos to days 5/6. Future studies comparing these two media systems in well-designed trials should be performed.'

The studies suggesting effects on mosaicism and follow-up cognition were judged to be of low quality. Further, the panel found no evidence of a difference between cleavage- versus blastocyst-stage transfer for cumulative pregnancy rates derived from fresh and frozen–thawed cycles following a single oocyte retrieval, but cautioned that the evidence for this outcome was very low quality.

Culture media – use and management/cold chain/storage

Culture media must be:

- stored refrigerated at 2–8°C (https://www.cdc.gov/vaccines/hcp/admin/storage/toolkit/index.html);
- kept away from light (and particularly direct sunlight);
- discarded when shelf life (i.e. expiry date) has been exceeded;
- labelled with expiry date and storage conditions; this information should also be on all product inserts;
- used in order of their lot/batch numbers

Light: To protect gametes and embryos from exposure to potentially damaging wavelengths of light, use filters in microscopes to block ultraviolet. Relative to microscope illuminations, the effect of ambient light stress is low, and this could be taken into account when planning a new laboratory.

Temperature and transportation:

- Any culture medium that has been frozen must be discarded.
- Culture oil will not freeze but oil can show cloudiness, which usually reflects reduced solubility of water or other molecules at low temperatures; this should be reversible at ambient temperature. If so, the oil does not need to be discarded.

Elevated temperature can lead to:

- Degradation through oxidation (relative loss of active ingredients).
- Amino acids and proteins may undergo deamination (ammonium ions; main sources of ammonia are unstable monopeptides of glutamine and protein sources).

Refrigerated storage of culture media (https://www.cdc.gov/vaccines/hcp/admin/storage/toolkit/index.html):

It was the consensus that IVF laboratories should use 'clinical grade' refrigerators with wire shelves for circulation, no closed bins, and no door shelves. The use of combined freezer/refrigerator units with no compartment control is discouraged. The efficiency of a refrigerator, in terms of maintenance of a stable temperature, is dependent on load, which should be in the range of 30–80% of its storage capacity.

Independent monitoring and logging of refrigerator temperature is vital, using a certified, calibrated thermometer, with remote monitoring and automatic alarm.

Equipment monitoring and validation

Independent continuous monitoring of critical parameters of laboratory infrastructure and equipment is desirable at the very least, and is mandatory for some accrediting/licensing authorities.

Validation that the equipment performs as per its specifications is normally performed by the manufacturer or their agent, at the time of installation ('Installation Qualification'). Verification of user requirement specifications and equipment performance is undertaken by the user after installation and before first use ('Operational Qualification') and again at regular intervals, including after servicing or repair ('Performance Qualification'). In these cases, the purpose is to establish that the equipment's performance is within accepted tolerance ranges of its specifications (*ISO 15189:2012*; *Mortimer and Mortimer, 2015*).

Culture medium validation and monitoring

There are different types of KPI that monitor different aspects of laboratory performance, as per the Vienna consensus (*ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine, 2017*).

Test equipment – calibration and certification

Test equipment for the laboratory and/or culture environment should be certified and calibrated according to the manufacturer's recommendations. In the absence of recommendations, calibrations should be conducted at least twice per year, or more often if required to ensure ongoing fitness for purpose.

Accurate pH measurements may be difficult to achieve due to calibration with buffers not designed for use at 37°C and therefore should be interpreted with caution. Point-of-care blood gas analysers with autocalibration function could provide more accurate measurements. It should be noted that blood gas analysers do not measure the pH of zwitterion-buffered media reliably.

Sperm preparation for IVF

Spermatozoa for ART must be separated from the seminal plasma environment not only as soon as possible after ejaculation (allowing for liquefaction), but also as efficiently as possible (*Björndahl et al., 2010*; *Mortimer, 2000*). Depending on intended use, the spermatozoa are then suspended in culture media either capable of supporting in-vitro capacitation (e.g. for IVF) or not (ICSI).

For IVF sperm preparations a balance is required between achieving in-vitro capacitation and avoiding premature acrosome loss and sperm senescence that can occur in men who are 'fast capacitators' when their spermatozoa are incubated for prolonged periods under capacitating conditions. So, if there will be a long delay before IVF insemination (e.g. >2.5 h) then the prepared spermatozoa could be resuspended in modified medium (e.g. HEPES-buffered) and held at ambient temperature until about 2.5 h before insemination and then washed and resuspended in fertilization medium and incubated in a CO_2 incubator for the final 120 min before inseminating the oocytes.

Spermatozoa being prepared for ICSI can be processed and resuspended using a sperm 'buffer' because capacitation is irrelevant for fertilization using ICSI. The preparation should be held at ambient temperature to minimize ROS generation (e.g. in a Styrofoam box to protect it from light and cold drafts) until the ICSI dish is prepared.

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Inter clinic comparison of morphokinetic variables

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Introduction

Intrinsic and extrinsic factors may affect the morphokinetics (developmental pattern/shape over time) of an embryo *in vitro* and these can be studied using time lapse imaging technology. For example, aneuploidy can delay blastulation (Campbell *et al*, 2013 a,b) and culture conditions, such as gas composition and culture media, have also been shown to have an impact on morphokinetics (MK) (Meseguer *et al* 2012 ; Ciray *et al*., 2012). For such reasons, the development of embryo selection algorithms in one clinic may not be transferrable to another setting, particularly if laboratory practice differs. Also, with a lack of consensus on time lapse annotation practice between clinics, inter-clinic differences in MKs, may even be artefactual. In a retrospective exercise, we previously demonstrated how a published time lapse model required modification in order to improve clinical outcome for CARE Fertility patients and was not directly transferrable (Best *et al* 2013).

Objective

As part of an embryo selection algorithm validation and implementation process, following the development of an algorithm in clinic M, we compared the distribution of five MK variables, used in our in –house developed models, from 859 ICSI blastocysts selected for transfer or vitrification. These variables were compared between three CARE Fertility clinics (L (15%), M (62%), N (23%)), with closely defined embryo culture practice and strict annotation policies, in order to establish whether the distributions of MKs were similar in selected embryos on three sites.

Materials and Method

The EmbryoScope™ time lapse imaging system and EmbryoViewer (Unisense Fertilitech, Denmark) was used to culture embryos, following ICSI, at 5%O_2, 5.5% CO_2 and 89.5N_2 at 37.0ºC.
Images were captured every 10-15 minutes through 7-9 focal planes during the culture period.
Images were studied by embryologists and the time points that the embryo reached developmental milestones, degree of fragmentation, evenness and anomalies were recorded (annotated).

Results table

Median values for the five variables and summary of differences between each pair of clinics (times in hours post ICSI).

	t2	t5	tSB	tB	CC2
Clinic L	24.5	47.8	95.7	107.8	10.5
Clinic M	24.5	48.5	96.2	105.9	11.2
Clinic N	24.9	48.8	95.2	108.9	11.2
Sig. diff. pairs	ns all pairs	ns all pairs	ns all pairs	L-N L-M (p<0.05)	L-N L-M (p<0.05)

MK variables compared

Time of ICSI to 2 cells (t2); 5 cells (t5); start of blastulation (tSB); full blastocyst (tB) and duration of second cell cycle (CC2). CC2=t3-t2.
The distributions (median and quartiles) were compared between each pair of clinics, using Wilcoxon signed rank test.

Figure 1

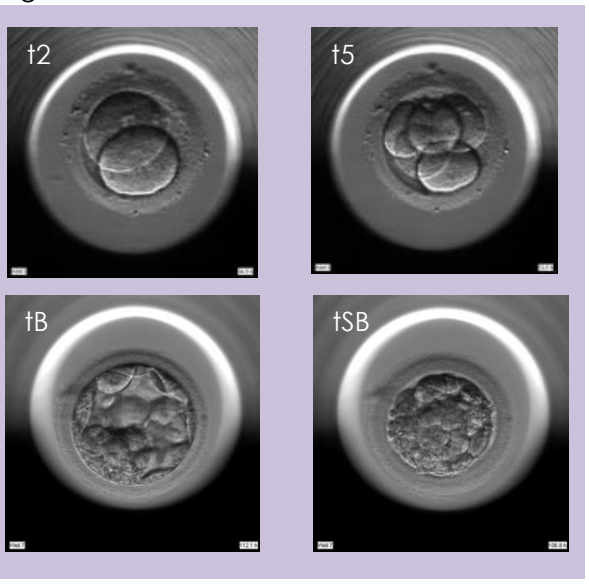

Figure 1. Time lapse images showing embryo appearance at study time points

Discussion

Performing inter clinic comparisons, along with retrospective testing of algorithms, is essential for effective use of embryo selection models and scrutiny of best clinical practice.

The cause of variation in tB and CC2 median values in clinic L has been considered and may be due to a difference in selection of embryos for cryopreservation in clinic L compared to M and N. MK distributions for all (unselected) embryos and those with positive and negative outcome will be compared in further studies with larger data and may highlight other possible causes, such as patient profile or annotation practice, and to fine-tune algorithms.

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7.0 General discussion

7.1 Progress in relation to specific aims

This thesis was largely successful in the pursuit of its specific aims, namely:

1. A retrospective time lapse analysis of euploid and aneuploid human embryos was performed and significant differences in their morphokinetic profiles noted. Although morphokinetics is not diagnostic of aneuploidy, a classification model to rank embryos according to their risk of aneuploidy was nonetheless developed.
2. The clinical relevance of this aneuploidy risk model was evaluated and put into clinical practice to aid non-invasive embryo selection with the aim of enhancing implantation and live birth rates.
3. Probably the largest body of quality assured time lapse data with clinical outcomes, to date, was collated during IVF treatments to make novel observations and develop in house embryo selection algorithms. Their efficacy in predicting implantation and live birth rates was established to show a significant improvement on conventional morphological selection, benefitting many thousands of patients.
4. A collaborative effort was undertaken to generate a multicentre outcome analysis that considered the limitations of a published and promoted time-lapse blastocyst prediction model. The main findings were that to adhere strictly to a specific algorithm predictive of blastulation could potentially result in the disposal of viable embryos, and that prediction of live birth was a more robust outcome measure to aim for.
5. Finally, I successfully published several book chapters and engaged with the scientific community in the development of guidelines for the nomenclature and annotation of dynamic human embryo monitoring. Significant outcomes were also a set of IVF

performance indicators, an "Atlas of time lapse embryology" and two consensus documents developing performance indicators and guidelines for IVF culture conditions.

At the outset of this thesis, the most commonly used methods of 'grading' embryos were according to a range of microscopic, morphological criteria that were either semi-quantitative at best or highly subjective at worst. Moreover, all had varying degrees of reported correlation to viability and, as such, were largely unsatisfactory (Centre for Disease Control and Prevention, 2014). The embryo, once *in vitro*, is an independent entity, separated from the complexity of female physiology and on a somewhat predetermined metabolic path, with its parental-derived genetic make-up, and its maternal cytoplasm providing some predetermining features. The work encapsulated in this thesis has made significant advances in the examination of several aspects of these elements within its defined culture environment, allowing for the prospect of a more meaningful and quantitative assessment.

The provision of such a quantitative assessment is perhaps the crux to the philosophy not only of this thesis, but of embryo selection in general. Essentially, we have two options: a binary option – the embryo is viable or non-viable; normal or abnormal. Or, a probability statistic; a ranking of one embryo over another, often based on putative data indicative of a particular propensity to implantation or delivery. A commentary by Mastenbroek and colleagues suggested that because all embryos can be frozen there is no need for embryo selection as this could "*never lead to improved live birth rates*" (Mastenbroek, et al 2011). They argue that as long as we can freeze and serially transfer all embryos – now that cryopreservation is efficient and endometrial receptivity is better understood – "*the live birth*

rate per cycle can never be improved". I would argue that the results herein encapsulated, largely dispel this notion. Efficient IVF is about maximising the chance of a live birth per single attempt – be that cycle started, egg collection or embryo transfer. Consideration must be given to patient welfare and most patients wish to avoid multiple visits to an IVF clinic – either for reasons of cost, social and emotional stress or all of these. Mastenbroek et al (2011) do, at least, concede that "*the only parameter that could possibly be improved by embryo selection would be time to pregnancy*". Time, as all practitioners of reproductive medicine are aware, is a valuable commodity, it is inversely related to reproductive efficiency – indeed, it should be reduced at all cost.

Selecting viable embryos at each attempt, therefore, ought to be beneficial to the patients for many reasons. Hence, for several decades, there has been determined work by scientists the world over to find ways to do this efficaciously. For embryo selection to be reliable and enduring, we require both the robust technology and its direct relevance to biological outcome to be established and constantly reassessed.

7.2. Thoughts on embryo selection

Arguably, the best means of embryo selection is non-invasive, either by harmless observation of the embryo itself or analysis of its environs. The information obtained should be precise, and preferably binary. Ranking is a more realistic approach but is always likely to create dilemmas and by definition reduced efficiency. Most biological data will inevitably be graduated, and therefore any ranking would need to generate highly correlative data for specific parameters. Ideally, data should be obtained independent of the influential reproductive tract. With all possible selection methods, patient cohort and the potential

dysfunction of the endometrium, impaired decidualisation and engaging theories on embryo selection by endometrial stroma (Salker et al, 2010; Teklenburg et al, 2010) will compound the analysis of singular screening events ranking embryo viability.

With regard to these criteria, we are currently faced with few useful technologies that can help practitioners make choices and patients achieve maximum success at each attempt. Currently, fully automated time lapse systems are not proven and validated as effective for embryo assessment and selection. There is, therefore, a reliance on manual assessment and annotation of time lapse image data, along with the possibility to derive algorithms in house (as described within this thesis) to provide a more precise, and non-invasive method of embryo assessment.

7.3 Second opinions and training

One of the key practical advantages of time lapse systems in the IVF laboratory is the facility to allow clinical embryologists to rewind, pause and review photographic frames in order to consider the detail and context of embryo development with flexibility and without interruption of embryo culture. The storage of images also allows them to be reviewed retrospectively in order to re-annotate or study in further detail. It can be particularly worthwhile to annotate additional variables, outside of standard operating procedure for a specific research interest or, for example, for embryos with known outcome only. This has been demonstrated within this thesis and has provided clinical relevance and aided the search for morphokinetic biomarkers for the ultimate outcome measure – healthy live birth.

7.4 Quality control and assurance

As with many aspects of the embryologist's role, there are practical and efficiency benefits if multiple individuals are trained and competent in routine daily tasks. Time lapse annotation is no exception and once introduced into the laboratory, review, annotation and interpretation of time lapse images can be considered a daily task. Where there are multiple practitioners involved, the risk of subjectivity and inconsistency is highest, although intra-practitioner annotation variation may also exist. Ensuring the most accurate and objective record of dynamic, often anomalous, embryo development brings challenges whether using automatic detection software, solo or collective manual methods. In order to minimise subjectivity, key variables for annotation were defined within the standard operating procedure (SOP) developed and used within the research studies described within this thesis. In general, SOP in IVF laboratories should be based on published best practice guidelines, adhered to, monitored and refined where required. Core morphokinetic variables need to be identified and annotated from the outset, following rigorous training and competency assessment. Some morphokinetic variables are more at risk of subjective interpretation than others; the appearance of pronuclei and time of compaction, for example. The use of reference images can be beneficial in assuring annotation quality.

The most commonly recorded morphokinetic variables follow the basic principles of embryology and mitosis and include timing of pronuclear appearance and fading, increasing cell numbers (time to 2, 3, 4, 5, 6 cells etc.) and times of embryo differentiation to the morula and blastocyst stages. Durations of mitotic cycles and synchronicity, can then be calculated from these. Additional to these, specific anomalies or associated phenomena can also be annotated, depending on the customisability of the time lapse system available. Once

established, regular audit of annotation completion and quality and adherence to SOP is essential in order to maintain high quality data to allow analysis and opportunity for identification of significant selection or deselection morphokinetic criteria.

There is evidence that many of the morphokinetic events are recorded objectively but it is critical that regular review and assessment exercises should be undertaken to assure quality. The work of Sundvall and colleagues demonstrated a close correlation between both experienced and new time lapse users for most morphokinetic variables but highlighted that some 'static morphologic parameters' such as multinucleation and blastomere evenness, remained at risk of subjectivity. With ongoing assessment, clear definitions and SOP, this can be minimised. In summary, best practice should ensure that time lapse culture dishes or slides are prepared in a standard and precise fashion, and that annotation of time lapse images, where performed manually, is performed with objectivity by all practitioners, audited and quality assured.

7.5 Flexibility and opportunity

Due to the great interest in time lapse technology by professionals in the field of IVF, promising clinical results, opportunities to broaden knowledge of the preimplantation embryo's development and its visual nature; which appeals to clinic staff and patients, swift advances in this technology and the application of it are inevitable. Computer servers, rather than standalone devices allow the support of new applications such as a patient interface, improved data collection, secure data storage, sharing and remote access.

Within this rapidly progressing and promising area of reproductive medicine, practitioners working with time lapse imaging have an additional and increasingly reliable tool for non-invasively studying the human preimplantation embryo and importantly - for improving embryo selection (Pribensky *et al*., 2017). Despite it making practical and scientific sense to utilise this technology which without intervention, enables the collection of vast image and kinetic data for analysis alongside clinical outcomes, time lapse imaging is considered new and unproven by many, and there are calls for more large-scale randomised controlled trials (Armstrong *et al*., 2018). One of the main challenges with such trials is the differing clinical, laboratory and embryo selection practice, which may introduce skew and affect reliability of the findings. Several large fertility clinic groups have published their own positive experiences using time lapse and developed selection algorithms which have been validated in house and are in clinical use. Several commercially available generic algorithms are also available through time lapse device manufacturers (Fishel *et al*., 2018; Peterson *et al*., 2016).

7.6 Morphokinetics and PGT-A

Time lapse technology alone and in conjunction with PGT-A, has the potential to provide continuously improving embryo selection algorithms which could incorporate numerous criteria resulting in algorithms defined for a range of varying circumstances, from individual patient criteria to generalised laboratory conditions. In time, the optimal ranges for defined dynamic events such as those directly associated with the 'normal', or euploid, cell cycle, may be elucidated and further novel morphokinetic markers of embryo viability identified (Gallego *et al*., 2019).

As mentioned above, the ultimate embryo selection tool will be reliable, reproducible, cost

effective, proven, non-invasive and have direct clinical relevance. When considering these ideals alongside standard morphological parameters, this routine practice is somewhat limited by subjectivity and reproducibility. IVF professionals continually search for alternative or complementary technologies to enable improved embryo selection and, given that chromosome constitution is critical to successful outcome, selection for euploidy is increasingly sought. The most reliable method for selection of euploid embryos is currently PGT-A. Several studies using time lapse imaging *in vitro* (including, significantly, in this thesis) have looked at the precise patterns and timings of preimplantation embryo development in relation to chromosome abnormality and studied whether aneuploid and euploid embryos have different morphokinetic profiles. As a result of this thesis and other studies, morphokinetic profiling has been proposed as a method to increase the probability of selecting chromosomally normal embryos, non-invasively, and also to classify an embryo's risk of aneuploidy.

As early adopters of time lapse for clinical IVF, Campbell and colleagues, considered whether there was a difference in the morphokinetics of euploid and aneuploid embryos, by comparing data from blastocysts which had undergone PGT-A (Campbell et al., 2013). This work did not identify any early morphokinetic markers associated with ploidy, as Chavez had done, and Basile went onto do. However, as is discussed in detail in section 2.0 of this thesis, from more than 20 variables compared, Campbell's group detected significant peri-blastulation delays in aneuploid embryos, compared with sibling euploid counterparts at the start of compaction (tSC), start of blastulation (tSB) and to the full blastocyst stage (tB). The authors devised an aneuploidy risk classification model based on their findings, incorporating the two most significant morphokinetic variables.

The clinical effectiveness of this model, was retrospectively evaluated for the potential impact on unselected IVF patients not undertaking PGT-A, as discussed in detail in section 3.0 of this thesis. In summary, following the transfer of 88 blastocysts, embryo fate, up to live birth, was compared according to calculated aneuploidy risk classes (low, medium, high). A significant difference was seen for implantation and live birth ratios between embryos classified with low and medium risk for aneuploidy, with relative increases of 74% and 56%, for embryos within the low risk class, compared to overall ratios, for foetal heart beat and live birth respectively (Campbell et al., 2013b). This study demonstrated, albeit on a small scale, the potential clinical relevance of such aneuploidy risk classification and introduced a novel, non-invasive method of embryo selection to yield higher implantation and live birth rates. This model was fine-tuned with a larger dataset and also assessed according to female age. This is discussed in more detail in section 3.0 of this thesis (Campbell *et al*., 2014).

There was some criticism of this aneuploidy risk classification model by Ottolini *et al*., who proposed that the delays observed in the aneuploid cohort were not the result of aneuploidy, but rather associated with maternal age (Ottolini *et al*., 2014). Campbell, however, published further analyses, in response, which described how age alone could not account for the different proportions of euploid embryos with advancing maternal age, and suggested that embryo ploidy is a key factor controlling human embryo morphokinetics. This debate is discussed in more detail within section 2.0 of this thesis (Campbell *et al*., 2014).

More recent larger scale studies described within this thesis further demonstrate that morphokinetic algorithm ranking correlates to blastocyst ploidy.

Figure 4 summarises an in house unpublished analysis of over 1500 PGT-A embryos at CARE

Fertility and demonstrates that top scoring 'A' embryos (according to the morphokinetic algorithm described within the published manuscripts detailed within this thesis) are significantly more likely to be euploid than the lower scoring embryos 'B' or 'C' (Figure 3).

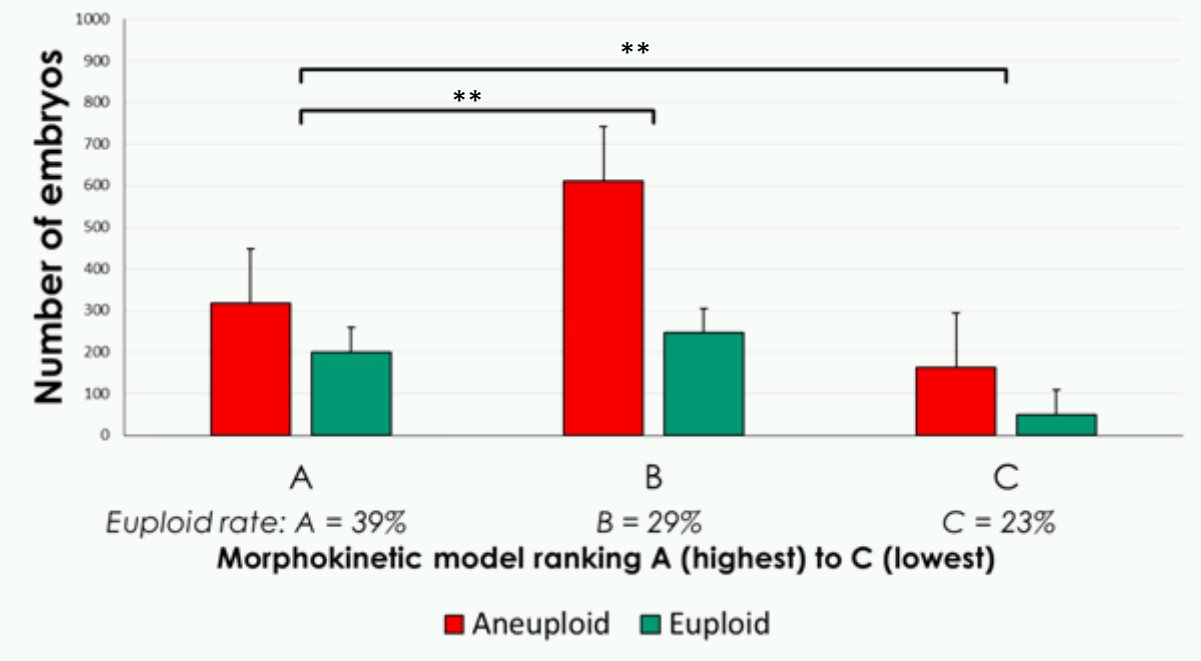
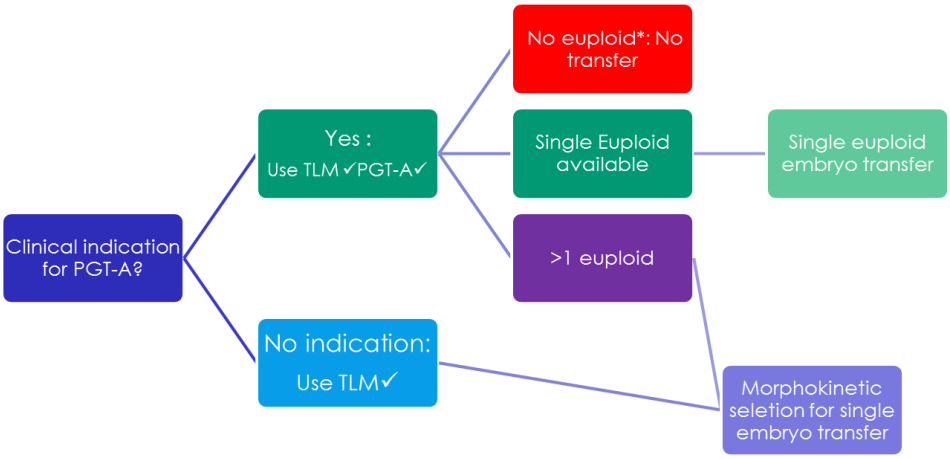


Figure 4: CARE Fertility unpublished data demonstrating that top morphokinetic grade (A) blastocysts are significantly more likely to be euploid than lower grades (B and C)

Time-lapse imaging is unlikely to ever be as absolute at elucidating ploidy from biopsied cells, or perhaps even by non-invasive PGT-A, but it is an important tool, which when utilised with precision and consistency, has been reported to enhance the chances of selecting an embryo, from a cohort, most likely to result in a live birth following IVF by non-invasive means (Fishel *et al*., 2019). It may also prove to be a useful tool for ranking or prioritising embryos for PGT-A, and for assisting in setting patient expectations, or for selecting between euploid embryos. Whilst euploidy is a requirement for optimal healthy live birth outcome, there are many additional factors necessary within the embryo itself, and maternally, to ensure successful

outcomes in ART and it is expected that time-lapse imaging, amongst other viability embryo assessment methods, will increasingly be used, in ART settings, in combination with PGT-A (Figure 5).



*Some circumstances may warrant mosaic embryo transfers

Figure 5: Proposed strategy incorporating PGT-A and time lapse monitoring (TLM) in ART

7.7 Future work

At CARE Fertility, we have compiled a database of over 500,000 fully annotated human embryos along with their fate (discarded, vitrified, transferred), and the clinical outcomes of over 10,000 time lapse annotated embryos, which have been transferred, and have a known outcome (implanted; ongoing clinical pregnancy; live birth; biochemical pregnancy; miscarriage). These data can also be linked to patient demographic, medical history, medication, laboratory treatment, providing huge potential to conduct further studies.

The future of IVF will almost certainly involve automation of time lapse image assessment, in real time, with degrees of certainty set by practitioners and used for discussions with patients and clinical decision making. It is hoped that this level of standardisation supported by large datasets will result in increased reliability in embryo selection and improved clinical outcomes, with shorter times to delivery of healthy babies for IVF patients.

Image analysis and artificial intelligence (AI) technologies are developing rapidly and it is likely that alongside automated assessment, semi or fully-automated AI objective algorithms will be introduced within IVF facilities over the next few years to further enhance clinical outcomes for patients by improving accuracy and objectivity.

7.8 Personal perspectives

When I was first introduced to time lapse imaging, and set about introducing it into the IVF laboratories which I oversee, I could hardly imagine how much it would impact patient treatments and our understanding of the nuances and patterns of the early human preimplantation embryo's development. The concept of time lapse is not new, it has been used by cinematographers for many decades, but it had not been available for routine clinical IVF until around 2011, when the EmbryoScope was introduced into our laboratory in Manchester. The first time lapse videos of embryos made a huge impression on the embryology team and wider staff, and the fact that the developing embryos remained undisturbed gave a more stable incubation, and enabled new flexible approaches in the laboratory. Patients were, and still are, thrilled to be able to receive time lapse videos of their embryos; starting their photo album of their baby sooner than ever before.

The main achievement of this thesis is that it has contributed to an evidence-based scientific framework through which time lapse monitoring can be structured and standardised in order to generate precise morphokinetic data, demonstrating differences in the development of viable embryos, upon which selection algorithms can be derived and applied to enhance outcomes for IVF patients.

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9.0 Appendices

9.1 Appendix 1

9.1.1 Log of media activity (radio, tv and articles) starting with the publication of the aneuploidy risk classification model described within section 2 of this thesis.

Date	Details	OrgName	StaffName
01/07/2011	EmbryoScope 1st UK Pregnancy	Manchester Evening News	Alison Campbell
04/07/2011	Embryoscope UK Pregnancy in Manchester	New Scientist	Alison Campbell
20/04/2012	Feature on first UK Embryoscope baby	Manchester Evening News	Alison Campbell
21/04/2012	First Embryoscope Babies	Daily Mail	Alison Campbell
21/04/2012	First Embryoscope Babies	Telegraph	Alison Campbell
23/04/2012	First Embryoscope Babies	Irish Independent	Alison Campbell
25/04/2012	News feature on embryoscope Manchester	Wigan Observer	Alison Campbell
26/04/2012	Embryoscope baby in Manchester	South Manchester Reporter	Alison Campbell
27/04/2012	Live interview on Embryoscope baby	BBC Radio 4	Alison Campbell
17/05/2013	CARE time-lapse research on BBC	BBC	Alison Campbell
17/05/2013	CARE Time Lapse Research Published	Telegraph	Alison Campbell
17/05/2013	Time Lapse IVF Research published	Guardian	Alison Campbell
27/10/2013	New sperm test off the shelf at Boots - CARE comment	Mail on Sunday	Alison Campbell
28/10/2013	New off the shelf sperm test kit - CARE comment	Telegraph	Alison Campbell
20/02/2014	CARE maps feature and photos	We Are Family	Alison Campbell
25/03/2014	First Mothers Day for Bradford Twins	Bradford & Argus Telegraph	Alison Campbell
25/03/2014	First Mothers Day for Bradford Twins	Bradford Telegraph & Argus	Alison Campbell
25/03/2014	First Mothers Day for Bradford Twins	Bradford & Argus Telegraph	Alison Campbell
25/03/2014	First Mothers Day for Bradford Twins	Bradford Telegraph & Argus	Alison Campbell
21/05/2014	CNN Documentary featuring CARE Maps and CARE Manchester family	CNN	Alison Campbell
17/06/2014	CNN Documentary promoting CARE maps again	CNN	Alison Campbell
17/06/2014	CARE maps referenced in time-lapse article	Bionews	Alison Campbell
17/06/2014	CARE maps referenced in time-lapse article	Bionews	Alison Campbell
07/03/2015	Interview with Kyle Casson and his mum	Scottish Daily Mail	Alison Campbell
08/03/2015	Interview with mum who gave birth to her grandson	Irish Daily Mail	Alison Campbell
11/03/2015	1000 Embryoscope Babies Born	Stroud Life	Alison Campbell
15/03/2015	Gloucester Citizen	Gloucester Citizen	Alison Campbell
17/06/2015	Video of Simon Fishel talking about CARE maps	Telegraph	Alison Campbell
06/07/2015	Access Fertility Package launch	BBC Look North	Alison Campbell
05/05/2016	Live evening interview on embryo research paper	BBC Radio Manchester	Alison Campbell
11/11/2016	Egg freezing article with Alison Campbell+	City AM	Alison Campbell
17/01/2017	BBC breakfast news discussing extension of 14 day embryo research rule	BBC	Alison Campbell
17/01/2017	Embryo research ruling extension from 14 days	BBC	Alison Campbell
03/03/2017	Alison Campbell on artificial embryo report from Cambridge	Channel 5	Alison Campbell
06/05/2018	Social egg freeze sell-by date should change	Daily Mail	Alison Campbell
10/09/2018	BBC Victoria Derbyshire Programme talking about male fertility	BBC	Alison Campbell
01/07/2019	Egg freezing feature Beacon CARE	Image magazine Dublin	Alison Campbell

9.1.2 The thesis author, Alison Campbell, with the UK's first baby in her arms and also on the screen (Bella) following the implementation of time lapse imaging for embryo selection (CAREmaps) at CARE Fertility, Manchester. 2012 and aged 2 and 4 years old.

9.1.3 Section from Reproductive BioMedicine Online Editor's report of 2017 showing the two Campbell articles described within this thesis (in sections 2 and 3) to be the most cited in 2016.

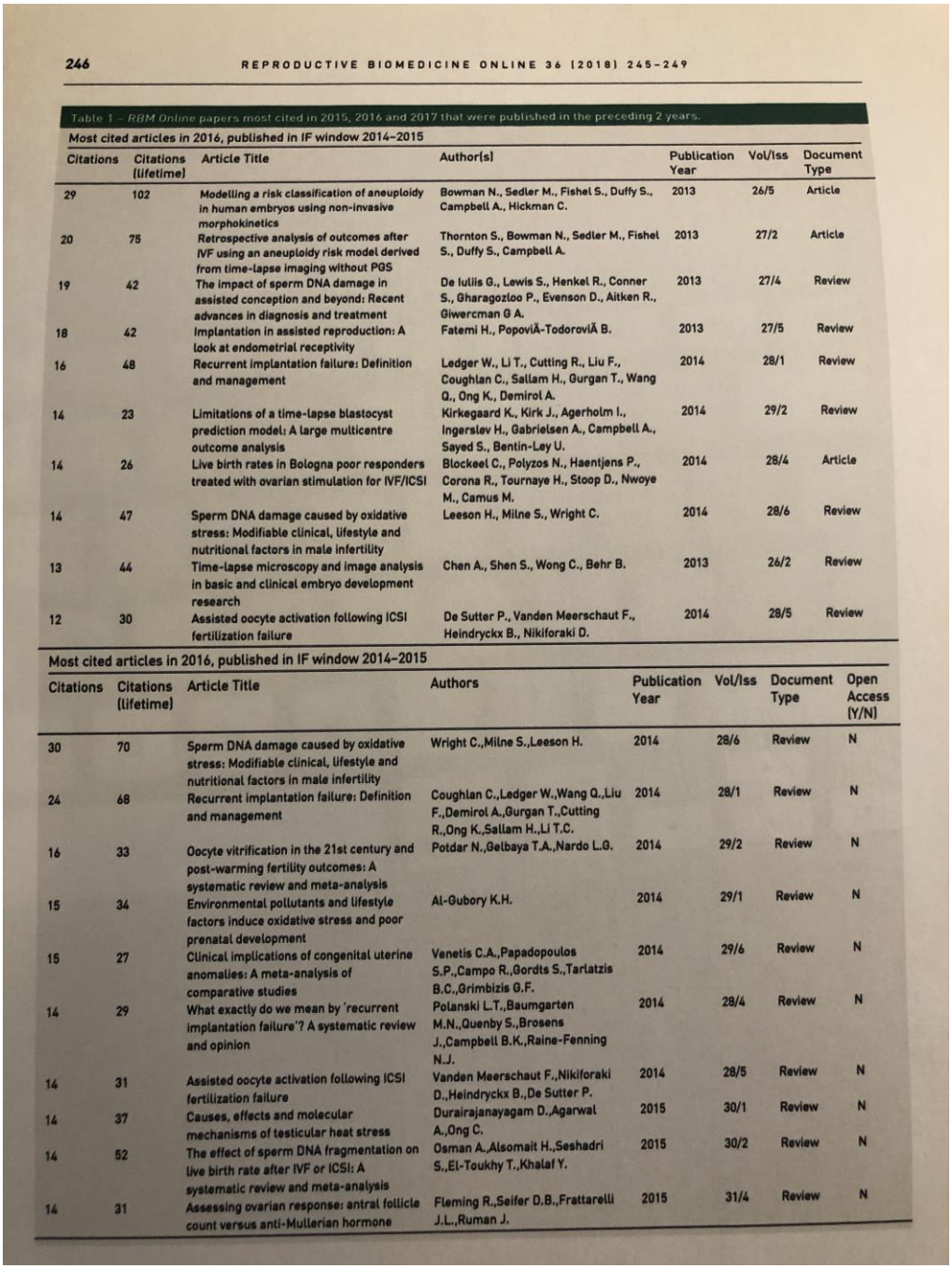

Table 1 – *RBM Online* papers most cited in 2015, 2016 and 2017 that were published in the preceding 2 years.

Most cited articles in 2016, published in IF window 2014–2015

Citations	Citations (lifetime)	Article Title	Author(s)	Publication Year	Vol/Iss	Document Type
29	102	Modelling a risk classification of aneuploidy in human embryos using non-invasive morphokinetics	Bowman N., Sedler M., Fishel S., Duffy S., Campbell A., Hickman C.	2013	26/5	Article
20	75	Retrospective analysis of outcomes after IVF using an aneuploidy risk model derived from time-lapse imaging without PGS	Thornton S., Bowman N., Sedler M., Fishel S., Duffy S., Campbell A.	2013	27/2	Article
19	42	The impact of sperm DNA damage in assisted conception and beyond: Recent advances in diagnosis and treatment	De Iuliis G., Lewis S., Henkel R., Conner S., Gharagozloo P., Evenson D., Aitken R., Giwercman G A.	2013	27/4	Review
18	42	Implantation in assisted reproduction: A look at endometrial receptivity	Fatemi H., PopoviÄ-TodoroviÄ B.	2013	27/5	Review
16	48	Recurrent implantation failure: Definition and management	Ledger W., Li T., Cutting R., Liu F., Coughlan C., Sallam H., Gurgan T., Wang Q., Ong K., Demirol A.	2014	28/1	Review
14	23	Limitations of a time-lapse blastocyst prediction model: A large multicentre outcome analysis	Kirkegaard K., Kirk J., Agerholm I., Ingerslev H., Gabrielsen A., Campbell A., Sayed S., Bentin-Ley U.	2014	29/2	Review
14	26	Live birth rates in Bologna poor responders treated with ovarian stimulation for IVF/ICSI	Blockeel C., Polyzos N., Haentjens P., Corona R., Tournaye H., Stoop D., Nwoye M., Camus M.	2014	28/4	Article
14	47	Sperm DNA damage caused by oxidative stress: Modifiable clinical, lifestyle and nutritional factors in male infertility	Leeson H., Milne S., Wright C.	2014	28/6	Review
13	44	Time-lapse microscopy and image analysis in basic and clinical embryo development research	Chen A., Shen S., Wong C., Behr B.	2013	26/2	Review
12	30	Assisted oocyte activation following ICSI fertilization failure	De Sutter P., Vanden Meerschaut F., Heindryckx B., Nikiforaki D.	2014	28/5	Review

Most cited articles in 2016, published in IF window 2014–2015

Citations	Citations (lifetime)	Article Title	Authors	Publication Year	Vol/Iss	Document Type	Open Access (Y/N)
30	70	Sperm DNA damage caused by oxidative stress: Modifiable clinical, lifestyle and nutritional factors in male infertility	Wright C.,Milne S.,Leeson H.	2014	28/6	Review	N
24	68	Recurrent implantation failure: Definition and management	Coughlan C.,Ledger W.,Wang Q.,Liu F.,Demirol A.,Gurgan T.,Cutting R.,Ong K.,Sallam H.,Li T.C.	2014	28/1	Review	N
16	33	Oocyte vitrification in the 21st century and post-warming fertility outcomes: A systematic review and meta-analysis	Potdar N.,Gelbaya T.A.,Nardo L.G.	2014	29/2	Review	N
15	34	Environmental pollutants and lifestyle factors induce oxidative stress and poor prenatal development	Al-Gubory K.H.	2014	29/1	Review	N
15	27	Clinical implications of congenital uterine anomalies: A meta-analysis of comparative studies	Venetis C.A.,Papadopoulos S.P.,Campo R.,Gordts S.,Tarlatzis B.C.,Grimbizis G.F.	2014	29/6	Review	N
14	29	What exactly do we mean by 'recurrent implantation failure'? A systematic review and opinion	Polanski L.T.,Baumgarten M.N.,Quenby S.,Brosens J.,Campbell B.K.,Raine-Fenning N.J.	2014	28/4	Review	N
14	31	Assisted oocyte activation following ICSI fertilization failure	Vanden Meerschaut F.,Nikiforaki D.,Heindryckx B.,De Sutter P.	2014	28/5	Review	N
14	37	Causes, effects and molecular mechanisms of testicular heat stress	Durairajanayagam D.,Agarwal A.,Ong C.	2015	30/1	Review	N
14	52	The effect of sperm DNA fragmentation on live birth rate after IVF or ICSI: A systematic review and meta-analysis	Osman A.,Alsomait H.,Seshadri S.,El-Toukhy T.,Khalaf Y.	2015	30/2	Review	N
14	31	Assessing ovarian response: antral follicle count versus anti-Mullerian hormone	Fleming R.,Seifer D.B.,Frattarelli J.L.,Ruman J.	2015	31/4	Review	N

9.2 Appendix 2

A selection of posters by the author and colleagues, associated with the work described within this thesis.

Assessment of early embryo development by the EmbryoScope™ (Unisense, Denmark) in relation to oocyte ploidy.

Alison Campbell[1], Samantha Duffy[1], Louise Best[1], Keith R Jordan[1], Natalie Bowman[1], Mark Sedler[1], Simon Fishel[2]

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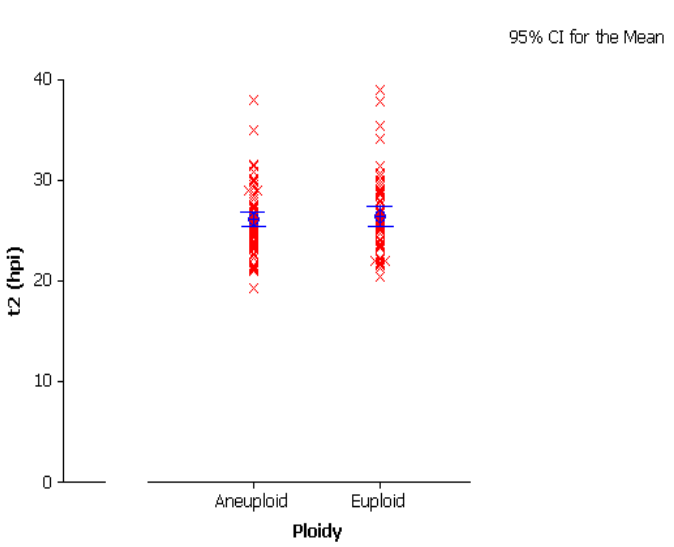
Abstract
Time lapse imaging of the preimplantation embryo allows embryologists to make more precise measurements of events in embryo development compared to the discontinuous methods most commonly used. The EmbryoScope™ was used to capture images every 20 minutes and in 7 focal planes throughout the culture period.

The time of the first cleavage to 2 cells (t2) was compared to oocyte ploidy following array CGH of the first polar body in 92 normally fertilised oocytes (2PNs). Of the 50 embryos developed from aneuploid oocytes, the mean t2 was 26.42hpi (min 19.27, max: 39.63). Of the 30 embryos developed from euploid oocytes, mean t2 was 26.38hpi (min 20.48, max 34.15). The polar bodies of the remaining 12 embryos did not amplify and so ploidy status was unavailable. Mean t2 for these was 25.87 (min 21.49, max: 30.48)
There appears to be no difference in the timing of the first cleavage of embryos developed from oocytes inferred as euploid or aneuploid following first polar body biopsy and aCGH. Our study continues the search for such non invasive temporal morphokinetic indicators of oocyte and embryo ploidy. (Further data has been added since abstract submission)

Time lapse monitoring using the EmbryoScope
The EmbryoScope exists of an in-built tri-gas incubator with HEPA and active carbon filters with a 20x HMC custom-made Leica objective providing a resolution of 3 pixels per µm. The EmbryoScope is connected to the Viewer; a Mac computer with EmbryoScope software and internet link.
The EmbryoScope allows 'continuous monitoring' of embryo development using time lapse microscopy at multiple focal planes. It can be set to take images at different time intervals and through different planes so that the user can view these images and focus through planes of the oocyte or embryo. Images are viewed individually as stills or as a video which can be rewound, played or paused. Annotation of morphokinetic events are performed by the Embryologist and these data collected and organised using the software.
The EmbryoScope accommodates up to 72 embryos - 6 patient 'slides' each containing up to 12 embryos in micro-wells approximately 250um in diameter.

Aim of the Study
To investigate whether there is a difference between the timing of the first cleavage (t2) following the fertilisation of oocytes inferred to be aneuploid or euploid, following polar body removal and array CGH.

Method
Following ICSI and first polar body biopsy, oocytes were placed into EmbryoScope slides and cultured in the EmbryoScope until embryo transfer or cycle cancellation. Time lapse images were viewed and annotated for first cleavage t2, and further developmental morphological and kinetic events, by an experienced embryologist and the data reviewed retrospectively and according to oocyte ploidy inferred by array CGH of the first polar body.



Results
Following ICSI and first polar body removal, normal fertilisation occurred in 166 oocytes (2PNs).
Of the 77 embryos developed from oocytes inferred to be aneuploid, the mean t2 was 26.14hpi (stdev 3.40, min 19.27, max: 37.96).
Of the 60 embryos developed from oocytes inferred to be euploid, the mean t2 was 26.43hpi (stdev 3.92, min 20.47, max 39.07).
The polar bodies of the remaining 29 embryos did not amplify and so ploidy status was unavailable. Mean t2 for these was 25.13 (stdev 4.36, min 7.97, max: 34.49).
Data were compared using 2 sample t-test.

Conclusion
There appears to be no difference in the timing of the first cleavage of embryos developed from oocytes inferred as euploid or aneuploid following first polar body biopsy and array CGH, p=0.7.
Our study continues the search for such non invasive temporal morphokinetic indicators of oocyte and embryo ploidy.

Acknowledgements – CARE Fertility Manchester Embryology Team and Cristina Fontes Lindemann Hickman.

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The use of live birth as an outcome measure for a new morphokinetic based blastocyst selection algorithm

Rachel Smith[1], Alison Campbell[1,3,4,5], Mette Laegdsmand[2], Sue Montgomery[3]; Louise Best[3]; Samantha Duffy[3], Davina Hulme [4], Devi Sivanantham[5], Lucy Jenner [4], Lynne Nice [5]and Simon Fishel [1,3,4,5].

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Introduction

Time lapse monitoring (TLM) allows the detailed comparison of morphokinetic variables between embryos, according to their fate following transfer. Data can be used to develop clinical evidence-based embryo selection algorithms. To date only a few embryo selection models have been published and it has yet to be established whether models are transferrable between centres. The only published morphokinetic model for blastocyst selection was based on aneuploidy risk classification (Campbell et al 2013). This new model introduces live birth outcome from transferred blastocysts.

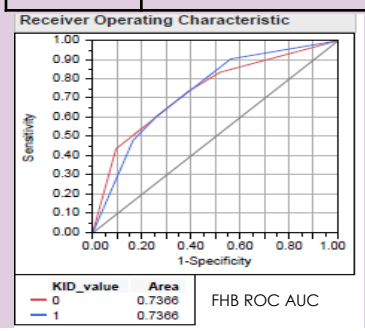
Participants/materials, setting, methods

Morphokinetic variables and outcome to fetal heart beat (FHB) n = 314 or to live birth (LB) n =80, for ICSI embryos following blastocyst transfer, were measured and annotated using EmbryoScope™ (Fertilitech, Denmark). Data were collected between May 2011 and August 2013. Time, in hours from pronuclear fading (pnf) to - all cleavage stages (relt2 to relt9); morula formation (reltM); start of blastulation (reltSB) and full blastocyst (reltB) were annotated using the EmbryoViewer software. The prefix 'rel' means that the variable is measured from PN fading to devise a model applicable to both IVF and ICSI. Where the number of transferred embryo(s) at least equaled the number of FHB(s) or LB(s), known implantation data (KID)of all embryos per treatment was positive, otherwise negative. KID rates were compared between model classes.

Results

An algorithm was derived using recursive partitioning of KID (based on FHB and LB) which ranked embryos according to their implantation potential calculated using KID rate (KID positive/(KID negative + KID positive) x 100. From 10 studied variables two were found to be significant reltM and reltSB. The Blastocysts were classified from A – C.

Grade	Model	FHB n	KID FHB rate	LB n	KID LB rate
A	Not grade C & reltM>= 52.6h and < 61.4h	89	64%	39	71%
B	Not grade C & reltM < 52.6 h or reltM >= 61.4H	128	39%	24	38%
C	reltSB>= 75.8h	97	11%	17	8%



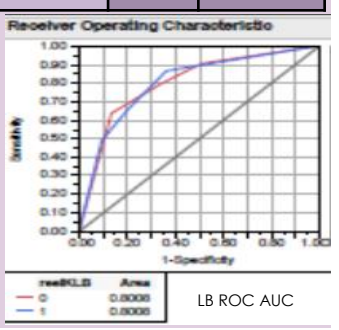
FHB ROC AUC

Area under the ROC curve

FHB 0.74

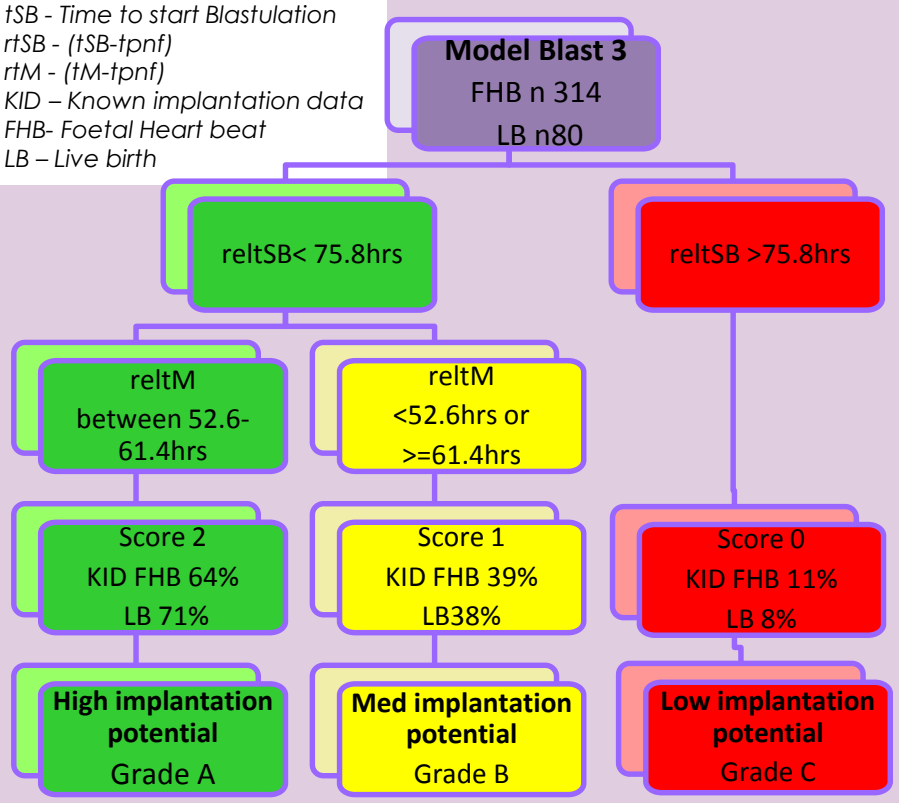
LB 0.80

Highly predictive



LB ROC AUC

Definitions
tpnf - Time to PN fade
tM - Time to Morula
tSB - Time to start Blastulation
rtSB - (tSB-tpnf)
rtM - (tM-tpnf)
KID – Known implantation data
FHB- Foetal Heart beat
LB – Live birth



Conclusion

Two morphokinetic variables (time to morula and start of blastulation both from PN fading) were found to have significant effect in a tree model of the implantation potential of transferred blastocysts, defined by foetal heart beat (FHB) and live birth (LB). The importance of reltSB corresponds to earlier published models where tSB was identified as a strong predictor for implantation potential (Campbell et al .,2013). The model is to be used for prospective clinical evaluation of embryos transferred after 96hrs post insemination. This model can be applied to both IVF and ICSI cycles by using the relative time from pronuclear fade widening the scope of the model. Care must be taken as selection models may not be transferrable to different clinic settings without adaptation (Best et al., 2013)

References:- Campbell, A., Fishel, S., Bowman, N., Duffy, S., Sedler, M., Hickman, C.F.L., 2013. Modelling a risk classification of aneuploidy in human embryos using non-invasive morphokinetics. Reprod. Biomed. Online 26, 477–485.
Best, L., Campbell, A., Duffy, S., Montgomery, S., Fishel, S., 2013.Session 57: Does one model fit all? Testing a published embryo selection algorithm on independent time-lapse data. Hum. Reprod.28, i87–i90.

Large scale comparison of morphokinetic timings of over 12,500 IVF and ICSI embryos from insemination to blastulation.

Alison Campbell[1]; Mette Laegdsmand[2]; Simon Fishel[1]; Rachel Smith[1]; Sue Montgomery[1]; Kathryn Berrisford[1]; Rob Smith[1]; Lynne Nice[1] and Darren K Griffin[3].

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Introduction

Time-lapse imaging has now been widely introduced in IVF clinics across the world. It is considered, by many, to be a superior tool, than morphology alone, for embryo selection and a recent randomised controlled trial has provided clinical validation (Rubio et al 2014). Several studies have compared the precise timings and durations, which embryos reach specific cleavage stages and developmental milestones, between transferred embryos resulting in successful pregnancy, or birth outcome, with those which failed to implant. Such morphokinetic studies have identified significant differences in timings of specific variables and identified these as candidates for embryo selection criteria and developed algorithms in order to improve the chance of selecting a viable embryo.

Information comparing developmental timings in IVF and ICSI embryos using time lapse imaging is limited although one study suggests that there exists an initial delay to early cleavage milestones, in IVF compared with ICSI, but that this is short-lived and that mean values for IVF embryos, reaching consecutive later cell stages, align with those of ICSI embryos after the 8 cell stage (Dal Canto *et al* 2012).

Time lapse imaging studies have demonstrated that ICSI embryos display earlier times ,from insemination, to first cleavage than conventional IVF embryos (Lemmen et al 2008; Dal Canto et al 2012). Such a difference could not be detected using standard IVF laboratory practice, of course. This difference in timings can probably be attributed to the time for fertilisation to occur; taking longer for IVF due to the time required for sperm binding and internalisation. An adjustment factor, to allow for this, if known, may provide additional accuracy and avoid current, and potentially flawed, practice which often involves comparison of absolute time-points (such as time to reach two cells (t2) or three cells (t3) between IVF and ICSI populations. An alternative approach, which has been utilised, may be to compare intervals between these time points (such as CC2; t3-t2) .

Methods – embryo imaging and culture

Morphokinetic data was retrospectively collected from 12,939 embryos using EmbryoScope™ time lapse imaging from multiple sites using standardised laboratory practice and culture conditions (5%O2; 5.5%CO2), between May 2011 and December 2013. Insemination times were recorded: IVF time when gametes were mixed and ICSI time midway through the ICSI procedure.

Figure 1: Schematic of morphokinetic variables from Atlas of Time Lapse Embryology

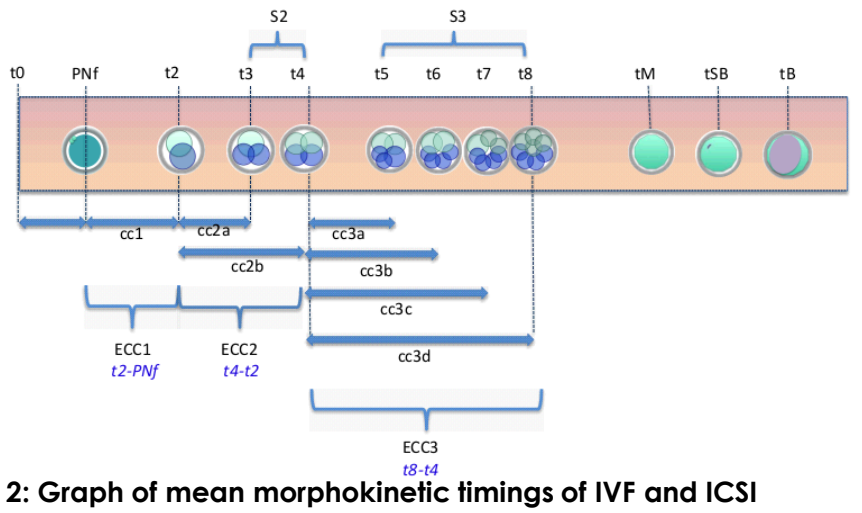
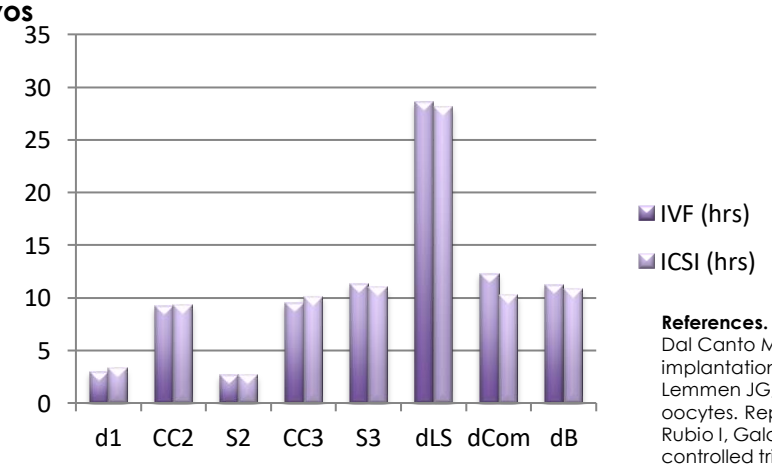


Figure 2: Graph of mean morphokinetic timings of IVF and ICSI embryos



Methods – Annotation of morphokinetics

Mean values in hours(h) and standard deviations were calculated for the following:
pronuclear fading (PNf);
time to 2 cells (t2) from PNf (d1);
Cell cycle lengths - t3-t2 (cc2a); t5-t4 (cc3a);
Synchronicity of divisions - t4-t3 (s2); t8-t5 (s3);
Duration of late stages (time to start compaction (tSC) from t8) (dLS);
Duration of compaction (dCom) and blastulation (dB).

Results

A difference in means of 2.3h between ICSI and IVF was observed in PNf. The mean (h) and standard deviation (in brackets) for ICSI and IVF PNf was 23.7(5.1) (n=9619) and 26.0(4.2) (n=3320) respectively. The delay was maintained as ongoing morphokinetics were similar , with no significances found, when comparing cell cycle, synchronicity and durations through to blastulation. Mean values and standard deviations were respectively as follows for
ICSI: d1 3.0(2.4); cc2 9.2(5.2); S2 2.7(4.8); cc3 9.5(6.6); S3 11.3(9.1); dLS 28.6 (11.2); dCom 12.3 (7.7); dB 11.2 (5.5) and
IVF: d1 3.3(2.6); cc2 9.3(4.8); S2 2.7(5.1); cc3 10.1(6.6); S3 11.0(9.1); dLS 28.1 (10.2) ;dCom 10.3 (6.3); dB 10.8 (5.0).

Conclusions

Morphokinetic algorithms, in order to be applicable to both IVF and ICSI embryos should avoid using time of insemination (t0), or direct derivatives of this, to assure accuracy. Durations, or relative time from pronuclear fading, provide more reliable variables for time lapse algorithm development considering the inevitable, but now measurable, delay in IVF embryos observed up to pronuclear fading.

References.
Dal Canto M, Coticchio G.,et al. Cleavage kinetics analysis of human embryos predicts development to blastocyst and implantation. Reprod Biomed Online. 2012;25(5):474-80.
Lemmen JG, Agerholm I, et al. Kinetic markers of human embryo quality using time-lapse recordings of IVF/ICSI-fertilized oocytes. Reprod Biomed Online. 2008;17(3):385-91.
Rubio I, Galan et al. Clinical validation of embryo culture and selection by morphokinetic analysis: a randomised controlled trial of the EmbryoScope. Fertility and Sterility. 2014: 102(5):1285-1294
Atlas of Time Lapse Embryology edited by Alison Campbell and Simon Fishel, CRC Press 2015. ISBN: 978-1-4822-1446-8

Ploidy, morphokinetics and time lapse imaging: The story so far.

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[2] Genetics, School of Biosciences, University of Kent, Canterbury. Kent, CT2 7NZ

Introduction

Time-lapse imaging has the potential to be either complementary or even an alternative option to PGS; it is reported to improve the chances of viable or euploid embryo selection. Here, we review, categorise and summarize scientific material (2012-2014) using citation databases and conference proceedings that consider the association between embryo ploidy and morphokinetic criteria, revealed by time lapse imaging. A brief overview of studies is provided and common or conflicting findings highlighted and discussed.
Aneuploidy in preimplantation embryos is a leading cause of IVF failure. Preimplantation genetic screening can be used to infer embryo ploidy following biopsy. However PGS has limitations of accessibility, acceptability, invasiveness and cost. Embryo morphology remains the most commonly used embryo selection criterion in IVF treatment. Non invasive markers of ploidy and viability are sought as only weak associations between morphology and ploidy have been reported to date.

Methods

21 relevant studies were identified.17 abstracts compared pre-compaction (early) parameters in euploid and aneuploid embryos. Seven abstracts additionally considered late morphokinetics ,according to embryo ploidy. Four original articles were identified.

Outcomes - Early morphokinetics (to 8 cells) (Fig.1)

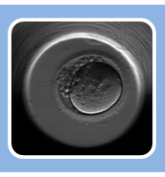
Five abstracts from 17 (29%) reported significant differences in morphokinetics of euploid and aneuploit embryos. Aneuploid embryos had increased multinucleation (2 studies); more irregular cleavage (one study); prolonged transition 2 to 4 cells (one study) and 5 to 8 cells (one study), compared with euploid. The remaining 12 (71%) abstracts reported no differences in early morphokinetics.
Two original articles considered early morphokinetic parameters of aneuploidy. One reported faster times to 5, from 2 and 3 cells in aneuploid embryos. The other concluded that cell cycle (prolonged first, and time between first and second divisions) and blastomere fragmentation parameters were diagnostic of ploidy.

Outcomes - Late morphokinetics (Fig.2)

7 abstracts additionally considered late morphokinetics. 5 (71%) reported peri-compaction and/or blastulation initiation and completion delays in aneuploid embryos compared with euploid, 2 (29%) found no differences. Significant peri-blastulation delays in aneuploid embryos were found in one original article but not in another.

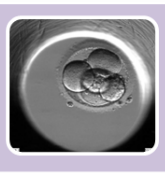
Difference reported are highlighted in the figures below

Figure 1. Reported early markers of embryo ploidy.



MULTINUCLEATION

- More MN (at 4 cell stage) in aneuploid embryos *(Melzer ASRM 2013)*
- Severe MN twice as high in aneuploid embryos *(Mazur et al ASRM 2013)*

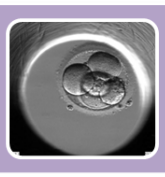


Outliers

Euploid embryos display strict /clustered cell cycle parameters. Aneuploid, more diverse.

Fragmentation – a response to aneuploidy?

(Chavez Nature Comms. 2012)



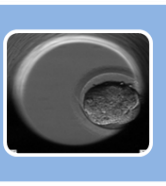
t5-t2 <20.5h, 10.4% euploidy; >20.5h, 34.4% euploidy

t5-t3 (cc3) Within 11.7-18.2h, 33.4% euploidy; outside window, 16.3% euploidy

cc2 and **t5** significantly different (faster in aneuploid)

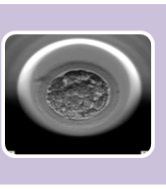
(Basile et al Fertil Steril 2014)

Figure 2. Reported late markers of embryo ploidy



START COMPACTION/CAVITATION 4-6h Delays

- Delays to tSC (p<0.05), and to tSB (p<0.01) tB (p,0.05) in aneuploid embryos. *(Campbell et al (RBM Online 2013)* (n=98)
- Longer duration to cavitation in aneuploid embryos (n=121). Times from 1st cytokinesis (P=0.02) or the 5 cell stage (P=0.01) to the onset of cavitation. *(Hong et al ASRM 2013)*



DURATION OF COMPACTION ~5h Delay

- <22h, significantly more likely to produce euploid blastocyst (p=0.009). Montgomery (ESHRE 2013)
- 5h delay - Longer duration of compaction in aneuploid embryos (p<0.004) (n=106) Melzer et al (ASRM 2013)
- Delays at Morula stage in aneuploid embryos (n=412). (Nagornyy et al , PGDIS 2014)

REACHING FULL BLASTOCYST, tB ~ 6h Delay

- Delays to tB (p,0.05) in aneuploid embryos. *(Campbell et al (RBM Online 2013)* (n=98)

Discussion

Findings from the multiple small investigations into ploidy and morphokinetics, presented as abstracts, are mixed. When considering early cleavage stage associations with ploidy, most studies did not report differences whereas, most of the abstracted data looking at later developmental associations did find delays in aneuploid embryos compared with euploid. Numbers remain small however and consensus has not been reached. Original articles, in this area, also remain few and findings are conflicting.

Associations between morphokinetics and ploidy require careful interpretation due to lack of consensus for definitions, variable PGS methodologies, sample sizes and time lapse devices used. Larger studies are needed but time lapse imaging remains a promising tool to enhance clinical outcome.

Full reference list available upon request

Assessment of early embryo development by the EmbryoScope™ (Unisense, Denmark) in relation to oocyte ploidy.

Alison Campbell[1], Samantha Duffy[1], Louise Best[1], Keith R Jordan[1], Natalie Bowman[1], Mark Sedler[1], Simon Fishel[2]

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[2] CARE Fertility, John Webster House, 6 Lawrence Drive Nottingham Business Park, Nottingham NG8 6PZ

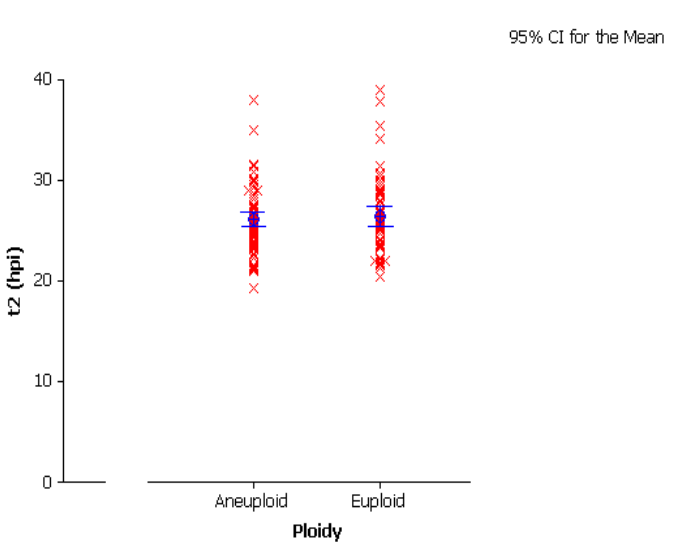
Abstract
Time lapse imaging of the preimplantation embryo allows embryologists to make more precise measurements of events in embryo development compared to the discontinuous methods most commonly used. The EmbryoScope™ was used to capture images every 20 minutes and in 7 focal planes throughout the culture period.

The time of the first cleavage to 2 cells (t2) was compared to oocyte ploidy following array CGH of the first polar body in 92 normally fertilised oocytes (2PNs). Of the 50 embryos developed from aneuploid oocytes, the mean t2 was 26.42hpi (min 19.27, max: 39.63). Of the 30 embryos developed from euploid oocytes, mean t2 was 26.38hpi (min 20.48, max 34.15). The polar bodies of the remaining 12 embryos did not amplify and so ploidy status was unavailable. Mean t2 for these was 25.87 (min 21.49, max: 30.48)
There appears to be no difference in the timing of the first cleavage of embryos developed from oocytes inferred as euploid or aneuploid following first polar body biopsy and aCGH. Our study continues the search for such non invasive temporal morphokinetic indicators of oocyte and embryo ploidy. (Further data has been added since abstract submission)

Time lapse monitoring using the EmbryoScope
The EmbryoScope exists of an in-built tri-gas incubator with HEPA and active carbon filters with a 20x HMC custom-made Leica objective providing a resolution of 3 pixels per µm. The EmbryoScope is connected to the Viewer; a Mac computer with EmbryoScope software and internet link.
The EmbryoScope allows 'continuous monitoring' of embryo development using time lapse microscopy at multiple focal planes. It can be set to take images at different time intervals and through different planes so that the user can view these images and focus through planes of the oocyte or embryo. Images are viewed individually as stills or as a video which can be rewound, played or paused. Annotation of morphokinetic events are performed by the Embryologist and these data collected and organised using the software.
The EmbryoScope accommodates up to 72 embryos - 6 patient 'slides' each containing up to 12 embryos in micro-wells approximately 250um in diameter.

Aim of the Study
To investigate whether there is a difference between the timing of the first cleavage (t2) following the fertilisation of oocytes inferred to be aneuploid or euploid, following polar body removal and array CGH.

Method
Following ICSI and first polar body biopsy, oocytes were placed into EmbryoScope slides and cultured in the EmbryoScope until embryo transfer or cycle cancellation. Time lapse images were viewed and annotated for first cleavage t2, and further developmental morphological and kinetic events, by an experienced embryologist and the data reviewed retrospectively and according to oocyte ploidy inferred by array CGH of the first polar body.



Results
Following ICSI and first polar body removal, normal fertilisation occurred in 166 oocytes (2PNs).
Of the 77 embryos developed from oocytes inferred to be aneuploid, the mean t2 was 26.14hpi (stdev 3.40, min 19.27, max: 37.96).
Of the 60 embryos developed from oocytes inferred to be euploid, the mean t2 was 26.43hpi (stdev 3.92, min 20.47, max 39.07).
The polar bodies of the remaining 29 embryos did not amplify and so ploidy status was unavailable. Mean t2 for these was 25.13 (stdev 4.36, min 7.97, max: 34.49).
Data were compared using 2 sample t-test.

Conclusion
There appears to be no difference in the timing of the first cleavage of embryos developed from oocytes inferred as euploid or aneuploid following first polar body biopsy and array CGH, p=0.7.
Our study continues the search for such non invasive temporal morphokinetic indicators of oocyte and embryo ploidy.

Acknowledgements – CARE Fertility Manchester Embryology Team and Cristina Fontes Lindemann Hickman.

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Inter clinic comparison of morphokinetic variables

Alison Campbell[1,2,3,4], Simon Fishel[1,2,3,4], Sue Montgomery[1], Lucy Jenner[2], Lynne Nice[4], Rachel Smith[3], Samantha Duffy[1], Davina Hulme[2] and Devi Sivanantham[4].

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Introduction

Intrinsic and extrinsic factors may affect the morphokinetics (developmental pattern/shape over time) of an embryo *in vitro* and these can be studied using time lapse imaging technology. For example, aneuploidy can delay blastulation (Campbell *et al*, 2013 a,b) and culture conditions, such as gas composition and culture media, have also been shown to have an impact on morphokinetics (MK) (Meseguer *et al* 2012 ; Ciray *et al*., 2012). For such reasons, the development of embryo selection algorithms in one clinic may not be transferrable to another setting, particularly if laboratory practice differs. Also, with a lack of consensus on time lapse annotation practice between clinics, inter-clinic differences in MKs, may even be artefactual. In a retrospective exercise, we previously demonstrated how a published time lapse model required modification in order to improve clinical outcome for CARE Fertility patients and was not directly transferrable (Best *et al* 2013).

Objective

As part of an embryo selection algorithm validation and implementation process, following the development of an algorithm in clinic M, we compared the distribution of five MK variables, used in our in –house developed models, from 859 ICSI blastocysts selected for transfer or vitrification. These variables were compared between three CARE Fertility clinics (L (15%), M (62%), N (23%)), with closely defined embryo culture practice and strict annotation policies, in order to establish whether the distributions of MKs were similar in selected embryos on three sites.

Materials and Method

The EmbryoScope™ time lapse imaging system and EmbryoViewer (Unisense Fertilitech, Denmark) was used to culture embryos, following ICSI, at 5%O_2, 5.5% CO_2 and 89.5N_2 at 37.0ºC.
Images were captured every 10-15 minutes through 7-9 focal planes during the culture period.
Images were studied by embryologists and the time points that the embryo reached developmental milestones, degree of fragmentation, evenness and anomalies were recorded (annotated).

Results table

Median values for the five variables and summary of differences between each pair of clinics (times in hours post ICSI).

	t2	t5	tSB	tB	CC2
Clinic L	24.5	47.8	95.7	107.8	10.5
Clinic M	24.5	48.5	96.2	105.9	11.2
Clinic N	24.9	48.8	95.2	108.9	11.2
Sig. diff. pairs	ns all pairs	ns all pairs	ns all pairs	L-N L-M (p<0.05)	L-N L-M (p<0.05)

MK variables compared

Time of ICSI to 2 cells (t2); 5 cells (t5); start of blastulation (tSB); full blastocyst (tB) and duration of second cell cycle (CC2). CC2=t3-t2.
The distributions (median and quartiles) were compared between each pair of clinics, using Wilcoxon signed rank test.

Figure 1

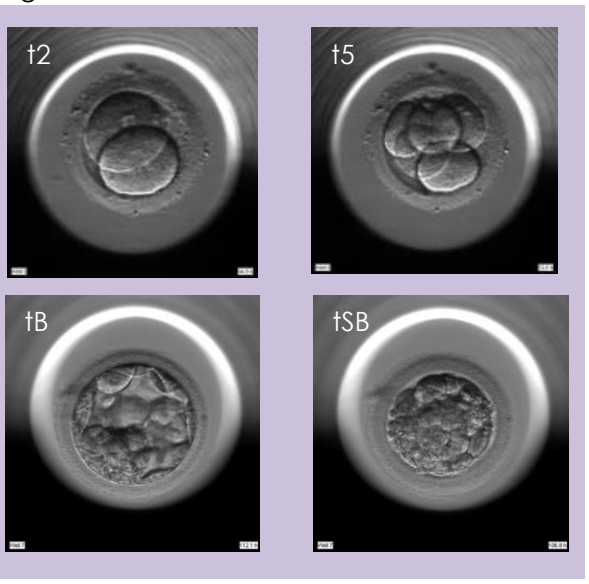

Figure 1. Time lapse images showing embryo appearance at study time points

Discussion

Performing inter clinic comparisons, along with retrospective testing of algorithms, is essential for effective use of embryo selection models and scrutiny of best clinical practice.

The cause of variation in tB and CC2 median values in clinic L has been considered and may be due to a difference in selection of embryos for cryopreservation in clinic L compared to M and N. MK distributions for all (unselected) embryos and those with positive and negative outcome will be compared in further studies with larger data and may highlight other possible causes, such as patient profile or annotation practice, and to fine-tune algorithms.

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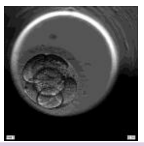
Acknowledgement – For statistical analysis support - Mette Laegdsmand, Unisense Fertilitech.

Comparison of morphokinetic variables of implanted and non-implanted embryos. A search for exclusion criteria.



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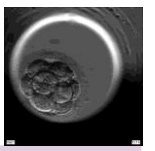

Introduction

Culturing human embryos in an incubator that incorporates time lapse microscopy enables embryologists to observe, record and analyse standard and novel developmental parameters without removing them from the controlled environment.
The EmbryoScope™ (Unisense Fertilitech, Denmark) is a tri-gas incubator with built-in camera, designed to automatically acquire images of embryos at specific time points. Images are taken several times per hour through multiple focal planes using a 20x Hoffman Modulation contrast objective. This novel incubation system enables simultaneous monitoring of up to 72 embryos with virtually uninterrupted culture conditions. Divisional events and morphological appearance can be reviewed and such variables annotated using the EmbryoViewer® workstation, enabling embryologists to compile a dynamic morphokinetic history of each embryo. These variables can be compared between transferred embryos with known implantation outcome in attempt to identify criteria which can be used to aid embryo selection.

The aim of this study was to compare morphokinetic variables of transferred embryos that resulted in clinical pregnancy/live birth and those which failed to implant, in attempt to identify criteria for embryo selection or exclusion, following ICSI.

Materials and Method

Data were obtained from *EmbryoScope™ ICSI treatment* at CARE Fertility, Manchester from May 2011 to October 2012. Following ICSI, oocytes were placed individually in microwells of the EmbryoSlide® in 25µl Global IVF medium (LifeGlobal®) supplemented with 10% Dextran serum supplement (Irvine Scientific), overlaid with 1.4ml mineral oil (Fertipro, Belgium) in the EmbryoScope™. These were cultured at 37°C in 5.5% CO_2, 5% O_2 and 89.5% N_2 for up to 6 days. Culture was interrupted for a few minutes on day 3 to refresh the medium for cases for extended culture. Data was grouped according to the time of ET. ET <96 hours post ICSI (hpi) was considered 'early' and >/=96hpi, 'late' for the purpose of this analysis.

Variables	Definition Table 1
t2	Time from ICSI to division to two cells completed (hours).
t3	Time from ICSI to division to three cells complete d(hours).
t4	Time from ICSI to division to four cells completed (hours).
t5	Time from ICSI to division to five cells completed (hours).
t8	Time from ICSI to division to eight cells completed (hours).
cc2	The duration of the second cell cycle (t3-t2) (hours).
cc3	The duration of the third cell cycle (t5-t3) (hours).
MN2	Multinuclearity at the two cell stage (true/false)
MN4	Multinuclearity at the four cell stage (true/false)
1->3	Direct cleavage from one to three cells (cc2<5hours)
2->5	Direct cleavage from two to five cells (cc3<5 hours)

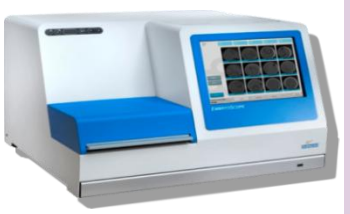

Figure 1: The Embryoscope™ and EmbryoSlide™ incubation trays

Image Acquisition and Annotation.

The built in microscope was used to acquire images of each inseminated oocyte every 10-20 minutes through 7 focal planes. The EmbryoViewer® image analysis software was used to log and display the precise timing and detail of developmental events both automatically and as they were annotated by the embryologists studying the time-lapse images. All annotations were made prior to embryo transfer and the outcome of treatment being known and therefore were blind to implantation outcome. Of 2365 standard ICSI embryos, **400 were transferred at <96 hpi and 121 embryos >/=96hpi.** The variables tabulated above in table 1 were compared.

Known Implantation Data value (KID)

KID was defined for each embryo from the number of successes (either as the number of gestational sacs (GS), number of foetal heart beat (FHB) or number of live born (LB)). The value of KID was 1 if the number of successes was equal to or exceeded the number of transferred embryos for that particular treatment. The value of KID was 0 if the number of successes was zero. The KID ratio (calculated as the number of embryos with known implantation/birth divided by the total number of transferred embryos with known implantation) does not reflect the overall success rate. KID-ratio is lower than the implantation rate with multiple transfers.

Results

Table 2. The mean implantation rate (IR) (calculated as the number of embryos that implanted to produce foetal heart beat divided by the number of embryos transferred) and the KID-Rs for early and late ETs.

ET	IR	n	KID (GS)	n	KID (FHB)	n	KID (LB)	n
< 96h	0.23	400	0.13	240	0.11	237	0.06	196
>/= 96h	0.43	121	0.44	96	0.41	96	0.21	62

Table 2

Table 3. The KID ratio and the number of embryos in each group of the early and late ETs for different groups of embryos defined by morphokinetic variables.

criteria	definition	ET < 96 hours			ET >= 96 hours		
		KID-R	n	p	KID-R	n	P
	all	0.12	208		0.37	67	
1	t2 <= 32 hours	0.13	192	0.03	0.41	94	0.51
	t2 > 32 hours	0.00	33	*	0.00	2	ns
2	cc2 >= 5 hours	0.14	168	0.04	0.42	83	0.55
	1->3 (cc2 < 5 hours)	0.05	64	*	0.31	13	ns
3	cc3 >= 5 hours	0.13	180	0.14	0.42	92	0.14
	2 ->5 (cc3 < 5 hours)	0.03	32	ns	0.00	4	ns
	not MN2	0.12	206	0.55	0.41	87	0.73
	MN2	0.06	31	ns	0.33	9	ns
	not MN4	0.11	232	1	0.40	96	-
	MN4	0.00	5	ns	-	0	ns
	selected (not fulfilling any of the criteria 1 or 2)	0.18	104	0.009 **	0.43	80	0.39 ns
	deselected (fulfilling criteria 1 or 2)	0.04	120		0.20	16	

Table 3

Fisher's test was used for significance of KID-R of embryos within and outside the group.

Discussion

Application of the exclusion criteria **t2 > 32 hours and cc2 < 5 hours** increased the KID-R from 0.12 (all) to 0.18 (selected) for early, and 0.37 to 0.43 for late, transfers. For early ETs, this gives a significant difference in KID-R between embryos deselected (excluded) by these criteria and those not. The difference in the late group is likely to be less significant due to the subset of embryos that reach this later developmental stage, in this better prognosis group. The improvement potential from using de-selection criteria may not be fully attainable in clinical practice due to lack of alternative embryos for some patients.

Acknowledgements: Mette Lægdsmand for data mining support, Unisense Fertilitech, Denmark.

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Does female age matter when selecting blastocysts for transfer using a novel morphokinetic based blastocyst selection algorithm?

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Introduction

The impact of female age on outcome of IVF is well documented and this has mostly been attributed to elevated rate of aneuploidy with increasing age (1). Aneuploidy has been linked with delayed development of blastocysts, and there is current debate regarding whether this may be confounded by age (2,3). Selection models based on the comparison of morphokinetic variables have been used to develop clinical evidence-based embryo selection algorithms which to date have not been age specific.

Study design

A novel morphokinetic selection algorithm was retrospectively applied to 795 transferred blastocysts of which 502 blastocysts had known implantation data (KID + or -) (May 2011 to March 2014). KID+ is defined by foetal heart at 6weeks +/- week.
Blastocysts were classified as low, medium or high implantation potential. KID+ rates (KID+/KID+ plus KID – x 100%) for each class, were compared between four age groups.

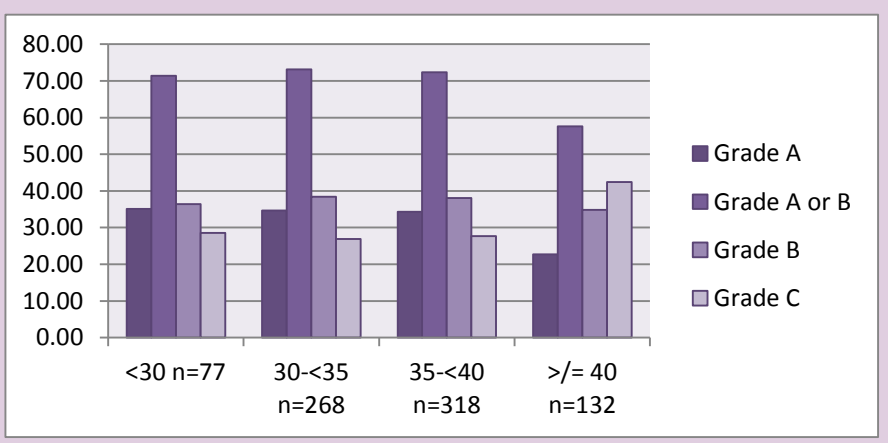
Blastocyst grading

Grade A blastocyst – high potential (n=159)
Grade B blastocyst – medium potential (n=185)
Grade C blastocyst – low potential (n=158)

Scored according to CARE Fertility in house algorithm based on durations from pn fading to time of morula stage and time to start blastulation.

Ages groups <30, 30-<35, 35-<40, >/=40

795 transferred blastocysts

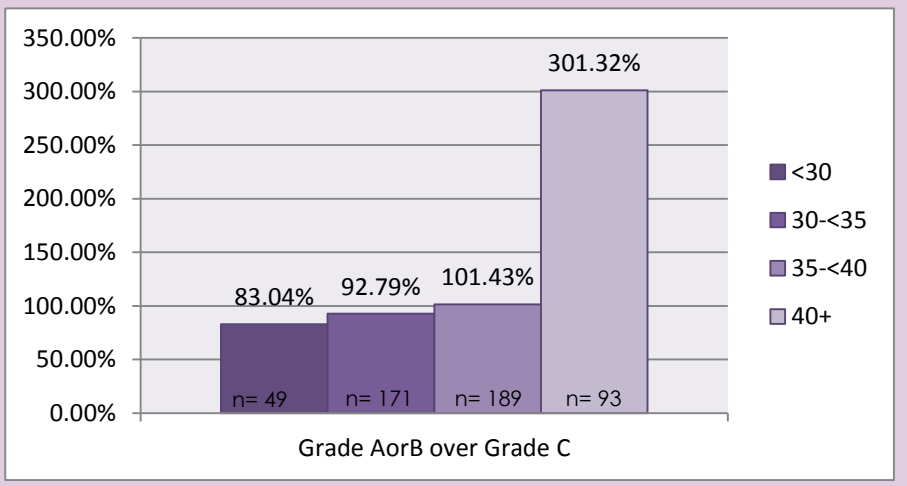


Probability of having a Grade C blastocyst transferred is significantly higher in the >40 group compared with the <35 age groups combined: 42.4% vs 27.2% (p=0.05).

There is no significant difference between the percentage of grade A+B blastocysts transferred between age groups.

Relative uplift in KID ratio with grading of embryo

From 502 blastocysts with known implantation



Transfer of grade A or B blastocysts, compared with transfer of grade C, demonstrated relative increases in all age groups, ranging from 83 to 301%. The relative uplifts increased with advancing maternal age groups, however the differences were not significant.

Conclusions and limitations

This work supports the use of a single morphokinetic blastocyst selection model, irrespective of age.
The outcome measure used to evaluate the potential impact of age on morphokinetics and hence to validate the effectiveness of a novel model, was blastocyst implantation. The optimal outcome measure is healthy live birth and further work is required to strengthen this data and evolve the model based on live birth.
Such models may not be transferrable between different settings and confounders may exist.
It is necessary to validate models prior to use and to consider potential confounding factors, such as age.

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Retrospective assessment of second polar body alignment using time lapse imaging

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Introduction

Early episodes of embryonic development have been suggested to be related to later embryo quality and pregnancy outcomes [1, 2]. One such episode is the extrusion site of second polar body (PB2) in relation to the first (PB1), which is not always adjacent [3]. In a previous study the magnitude of the angle between PB1 and PB2 at fertilization check showed no significant difference for embryo morphological grade on day 2 after ICSI or IVF [2]. Another study suggested that time of PB2 extrusion is important for embryo implantation on day 3 transfers, but the position of extrusion is not [3].

This study aims to analyse the relative position of PB2, at the time of extrusion, to PB1 and assess if it could be used to predict embryo development and implantation potential.
Our results demonstrate that an increased distance between PB1 and PB2, at extrusion, is correlated with poor embryo quality morphokinetic markers, and with decreased positive hCG and clinical pregnancy (FHB) rate per embryo transferred.

Methods

Blinded retrospective analysis of bi-dimensional time-lapse imaging was performed on 212 embryos (141 ICSI cycles) with known implantation data (KID) (positive and negative), 120 embryos (56.6%) were transferred on day 3 (D3) and 92 (43.4%) on day 5 (D5) (Table 1). The study did not exclude any form of infertility presentation or age.
Estimated measurement, using photographic time lapse images of the distance between PB1 and PB2 was recorded at the time of PB2 extrusion (PB2ext), pronuclear appearance (PNa), and pronuclear fading (PNf). Embryos were divided into four groups (pictorially demonstrated in figure 1).

- **1 = PB2 adjacent to PB1**
- **2 = PB2 ≤ 45° to PB1**
- **3 = PB2 between 45°-90° to PB1**
- **4 = PB2 ≥ 90° to PB1.**

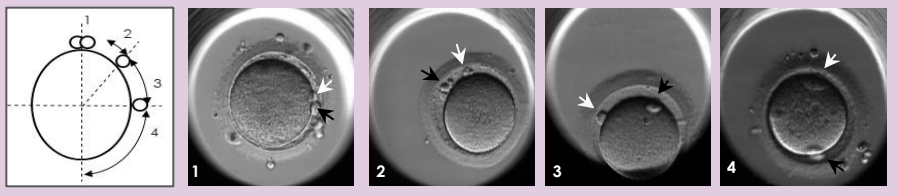

***Figure 1**; Diagram of measurement of distance between PBs. Examples of oocytes in each group depending of PB2 alignment to PB1.* Black arrows indicates PB1; White arrows indicates PB2.

Kinetic events such as time of pronuclear fading (PNf), time to two cell (t2), second cell cycle (CC2), time to four cell (t4), time to start of blastulation (tsB) and time to blastocyst (tB), embryo grading based in morphokinetic parametres described in Campbell *et al.* (2013), and implantation were compared between the four groups. ANOVA, Student's t-test and $\chi 2$ test were used to determine significance. $P < 0.05$ was considered to be significant.

Conclusion and discussion

The current study has demonstrated that, in a two-dimensional profile, but using focal planes to assess, embryos with a greater distance between PB1 and PB2 tend to have specific delayed morphokinetic previously associated with reduced embryo viability, and lower implantation rates at blastocyst stage. Further data is needed to consider whether this parameter can be incorporated into an embryo scoring systems or morphokinetic selection model.
As only embryos of known implantation were analysed, the number of poor quality embryos is low in this cohort. A larger cohort is needed to clarify significance.

Results

The majority of PB2 were extruded within 45° of PB1. A non significant trend was observed when data was analysed for maternal age (Table1).

PB alignment groups	1	2	3	4	NA	P val
N embryos (%)	124 (58%)	49 (23%)	21 (10%)	4 (2%)	14 (7%)	
Mean age ±SD	36.2 ±4.86	37.44 ±4.94	37.66 ±5.44	39.46 ±2.69		0.22
D3 ET	68	31	12	2		
D5 ET	56	18	9	2		

***Table 1;** Number of embryos in each group at PB2 extrusion; mean age of woman in each group; number of embryos transferred on D3 and D5.*

Embryos with a greater distance between PB1 and PB2 at PB2 extrusion showed a significant delay at tSB ($P = 0.001$) and tB ($P = 0.004$)(figure 2). Adapted embryo score based on morphokinetic parameters as described in Campbell *et al*. (2013) showed to be lower on day 5 for embryos whose PB2 was extruded more than 45° from PB1 ($P < 0.001$) (Figure 3). Also the analysis of PB2 movement demonstrates that embryos whose PB2 moved more than 45° following the extrusion have lower score on day 3 ($P = 0.049$) compared to these that do not (Fig 4).
A significant difference between positive hCG rate ($P = 0.02$), and FHB per embryo transferred ($P = 0.02$) on embryos transferred at the blastocyst stage, was observed as the distance of PB2 increases from PB1 (fig 5).
It was also noted that as distance between PB1 and PB2 increases, there was a trend to delayed tPNf, t2, t4, and shortened CC2.

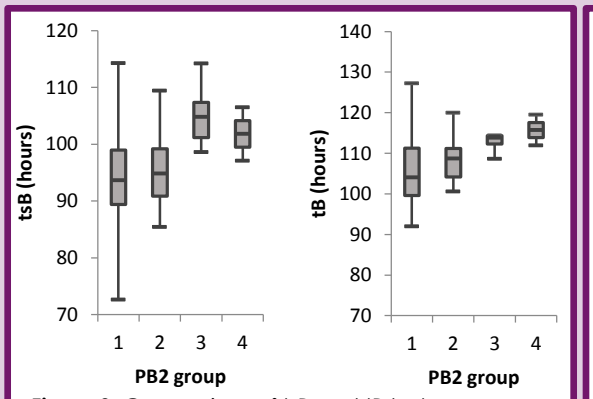

***Figure 2**; Comparison of tsB and tB between embryos in each PB2 group.*

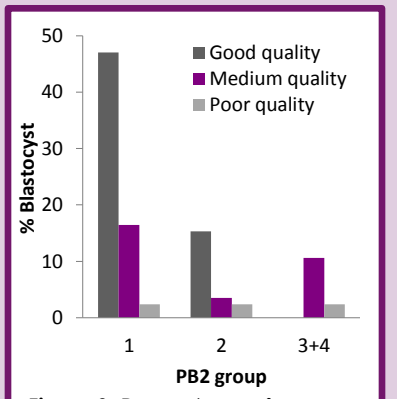

***Figure 3**; Percentage of different quality blastocyst for embryos in each PB2 group.*

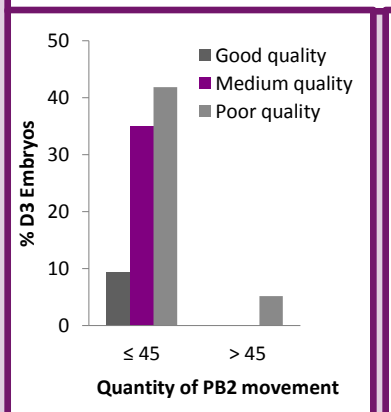

***Figure 4**; Percentage of different quality embryo on D3 depending of quantity of PB2 relative movement.*

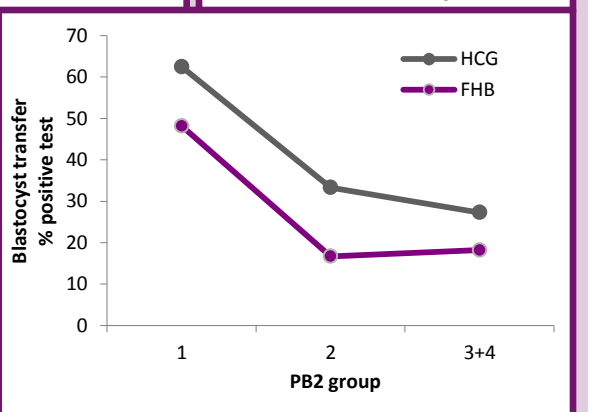

***Figure 5**; Percentage of positive results of hCG and FHB after blastocyst transfer for embryos in each PB2 group.*

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Effective use of preimplantation genetic screening following thawing, biopsy and re-vitrification of previously untested cryopreserved embryos.

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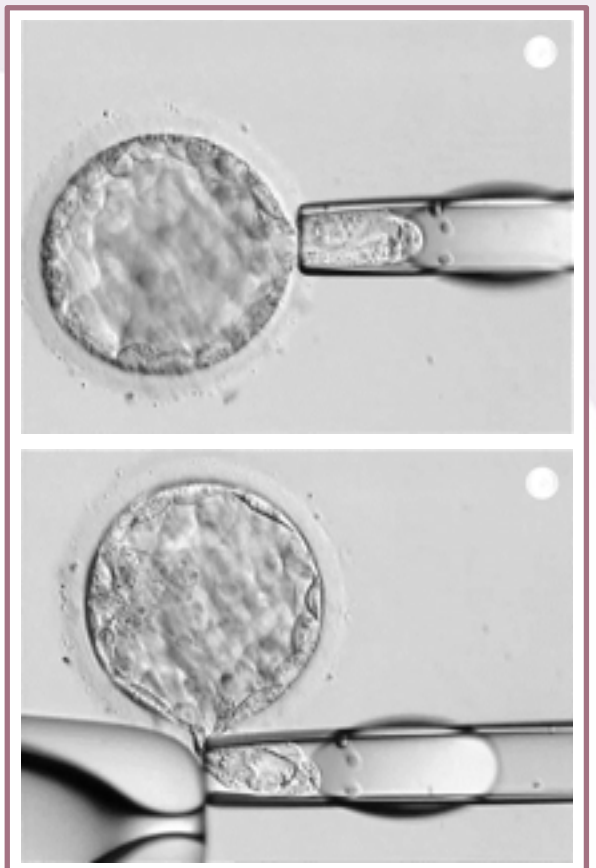
Introduction

Preimplantation genetic screening (PGS) has become a popular option for patients undergoing assisted reproductive treatment. Several studies showed that selecting euploid embryos for transfer, increases success rates, and reduces the risk of miscarriage and chromosomal abnormalities where clinically indicated.
Recently, there has been an increasing interest for patients to perform PGS on embryos frozen in previous cycles, when PGS was not available or it was considered not necessary. However such a technique has not been implemented routinely in IVF clinics.
This study analyses the effectiveness of performing biopsy on cryopreserved embryos.



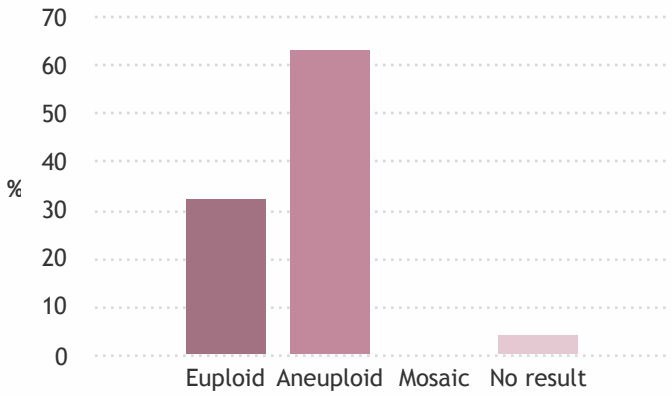
Images of trophectoderm biopsy.

Method

207 blastocysts from 59 patients were warmed for PGS from January 2016 to August 2017. If embryos survived, trophectoderm biopsy was performed as per routine protocol, and embryos were re-vitrified post-biopsy.
Samples were analysed for all the chromosomes with Next Generation Sequencing (NGS) and euploid embryos were replaced in single embryo transfers on subsequent 'frozen' cycles.

Outcome

196 blastocysts (94.7%) survived the warming process and were considered suitable for biopsy.
96% of the samples yielded conclusive PGS results, and 4% failed to give a result due to DNA quantity or quality. 63 of the embryos were reported euploid (32%).
To date, 25 euploid embryos have been warmed, all survived, and were replaced in single embryo transfers, resulting in 13 clinical pregnancies (52%).



PGS results with NGS after trophectoderm biopsy on

Discussion

This small study suggests that PGS may be effective in enhancing selection of cryopreserved embryos. Our results support previous publications (1, 2) showing that double vitrification and biopsy does not seem to be detrimental. PGS on frozen embryos could be a viable option for patients with recurrent implantation failure, or for patients with a large number of embryos cryopreserved, with the aim of reduce the number of embryo transfers required to achieve a live birth.

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Time lapse analysis of 1185 human blastocysts resulting in live birth to assess whether gender affects the timing of preimplantation embryo development.

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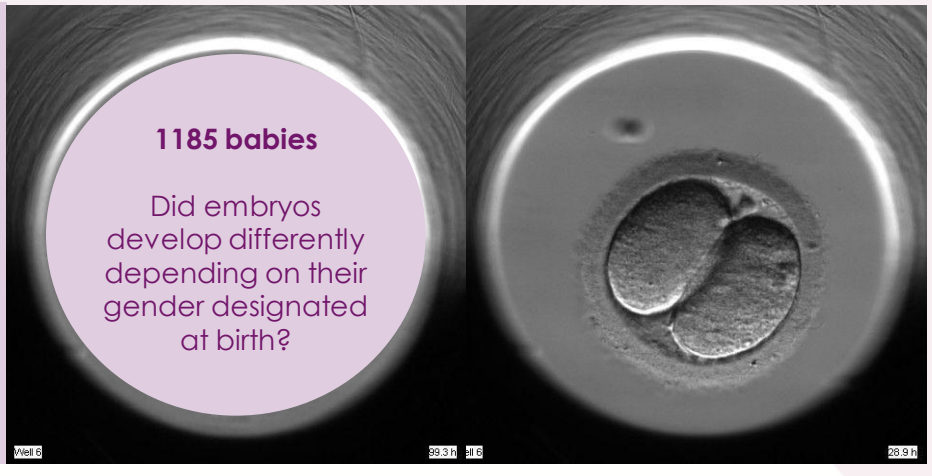
Introduction

Several non time-lapse studies have reported faster human preimplantation in vitro development of male embryos compared with female, whilst others found no difference. However, studies have been limited by static daily, as opposed to frequent dynamic assessments, possible with time-lapse monitoring.
Published time-lapse studies comparing morphokinetic timings, by gender are few, have contrary findings and are limited by the size of the datasets.



Methods

Using EmbryoScope (Vitrolife, Sweden), all embryos were cultured and assessed similarly, using strict and quality assured time-lapse annotation practice and were selected for blastocyst transfer according to standard laboratory protocol.

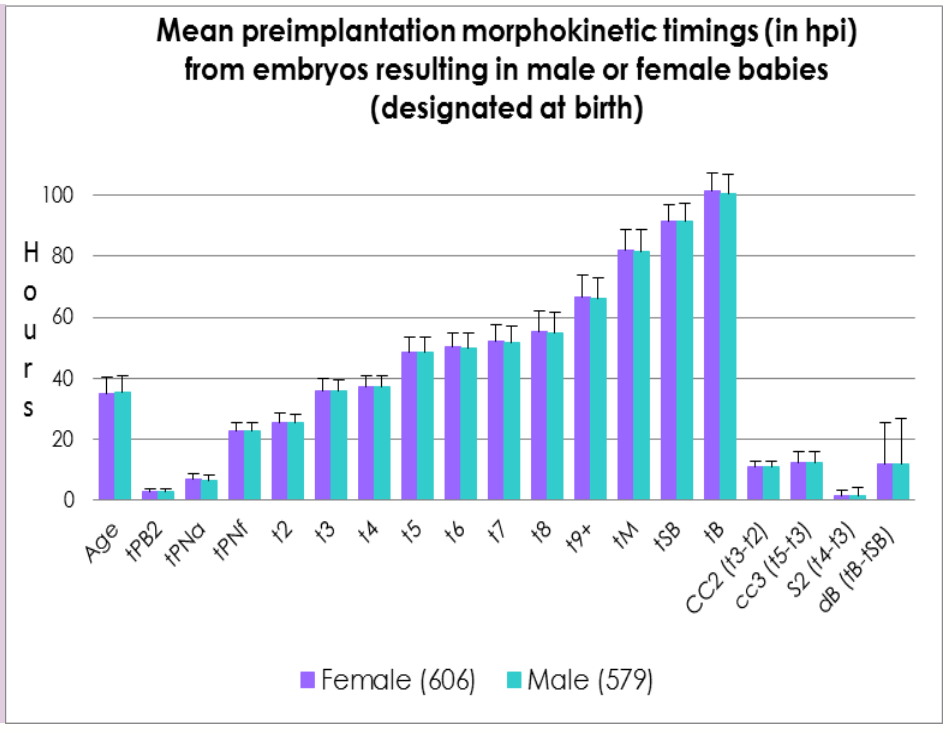
Mean timings for 14 preimplantation morphokinetic variables of 1185 singly transferred blastocysts, from an unselected IVF patient population, resulting in **606 (51%) female and 579 (49%) male babies** were retrospectively compared according to gender designated at birth. T-test was used to assess significance.

Results

Definitions and mean timings ±sd, in hours post insemination, (female; male) were: Extrusion of second polar body [2.93±0.93; 2.91±0.93]; pronuclear appearance [6.79±1.73; 6.61±1.72]] and fading [22.64±2.62; 22.71±2.67]; two to nine cells [t2: 25.40±3.23; 25.28±3.05], [t3: 36.07±3.70; 36.03±3.55], [t4: 37.15±3.50; 37.04±3.70], [t5: 48.32±5.32; 48.41±5.09], [t6: 50.21±4.84; 50.2±4.70], [t7: 52.23±5.51; 51.68±5.34], [t8: 55.18±6.97; 54.66±7.17], [t9+66.68±7.28; 66.17±6.95]; morula [81.90± 6.68; 81.58± 7.32]; start blastulation [91.35±5.69; 91.43±5.86] and full blastocyst [101.25±5.88; 100.74±5.98].

72.2% of female and 69.4% of male babies resulted from ICSI. Mean patient ages delivering either female or male babies were 35.1±5.24 and 35.45±5.17 years.

No significant differences were found between any of the morphokinetic variables assessed



Conclusion

This large retrospective analysis of gender specific human preimplantation development indicates that gender does not impact morphokinetics timings in vitro.

References

Bronet F et al. 2015. Is there a relationship between time-lapse parameters and embryo sex? Fertil Steril 103 (2) 396-401

Serdarogullari M et al. 2014. Comparison of gender-specifc human embryo development characteristics by time-lapse technology. RBMO 29, 193-199

CAREfertility

Alison.campbell@carefertility.com

Morphokinetics of 'Aneuploid' and 'Euploid' human embryos, inferred by polar body or trophectoderm biopsy

A. Campbell [1], CFL. Hickman [2], N. Bowman [3], S. Duffy [3], K. Gardner [3], S. Fishel [1]

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[2] *Trinidad and Tobago IVF and Fertility Centre, Trinidad and Tobago*
[3] *CARE Fertility Manchester, 108 Daisy Bank Rd, Victoria Park, Manchester M145QH*

Objective

To determine whether the ploidy of an embryo, determined by trophectoderm or polar body biopsy, affects its morphokinetics and development.

Materials and Methods

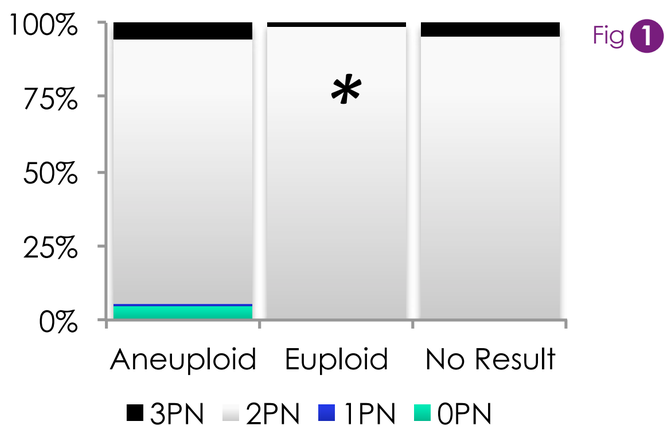
Following ICSI, 339 zygotes were cultured in a time lapse incubator (Embryoscope, Unisense, Denmark). The mean female age was 39+/-4 years. The morphokinetic timings [i.e. mean timing of second polar body (PB2) extrusion, cleavage to two-, three-, four-, five-, six- and eight-cell stage, start of compaction, formation of morula and blastocyst, and hatching (hpi: hours post insemination)] was compared between embryos reported as aneuploid (n=167) or euploid (n=119) following Preimplantation Genetic Screening (PGS) by trophectoderm biopsy or polar body biopsy using either array CGH or SNP microarray analysis. 'No result' was defined as ploidy being unreported by the analysing laboratory. Where embryo ploidy was stated, this was inferred from oocyte or embryo ploidy, following polar body or trophectoderm biopsy, respectively. The second cell cycle (cc2) of an embryo was defined as the time it reached 3 cells minus the time that it reached 2 cells. Blastocyst rate was calculated as the proportion of cleaved embryos that reached blastocyst stage by 144hpi. Normal embryo development was defined as sequential cleavage pattern with t5<70hpi, abnormal development was defined as very short second or third cell cycles (<5hours) and slow was defined as undergoing first cleavage or cleavage to 5 cells (t5) very late (>32 and >70hpi respectively). Embryos with slow or abnormal developmental patterns were removed from the data for analysis in fig 4 and 5 below, due to their distortive effect on the means. Data were presented as mean hpi+-standard deviation and the means for each stage of development were tested for significance using the paired t-test. Proportion data were tested for significance using the chi-square test.

* significant difference.

Results

1. Two pronuclei were observed in 89% of Aneuploid embryos, 99% of Euploid embryos and 95% of 'No result' embryos (p=0.006)

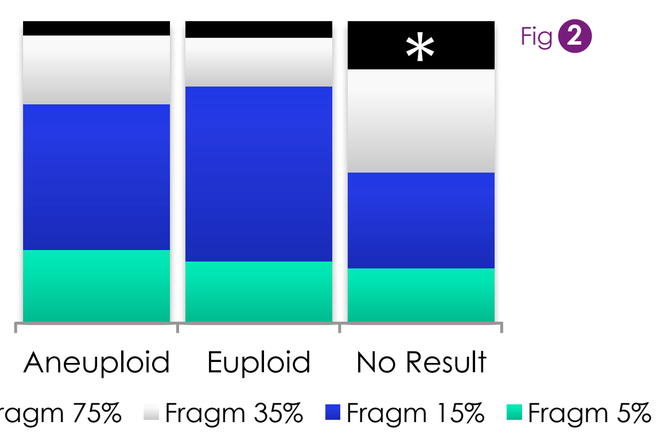
Fig 1



The proportion of 'No Result' embryos with:

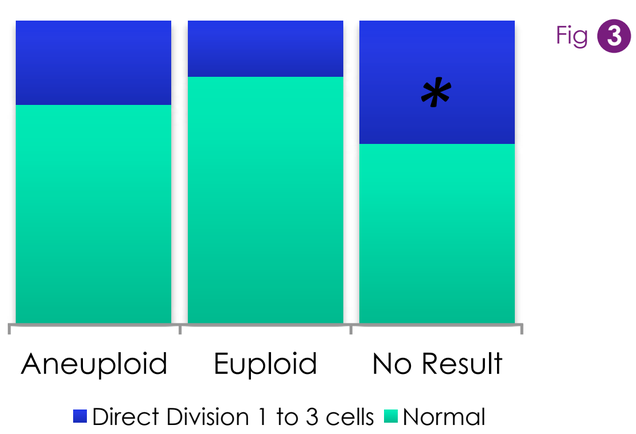
2. More than 75% fragmentation was greater (16%, n=44, p<0.05) than Aneuploid (5%, n=128)or Euploid (5%, n=93) embryos

Fig 2



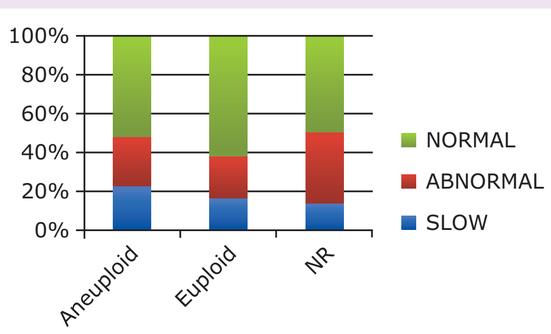
3. Direct division from 1 to 3 cells was greater(40%, n=42, p=0.03) than Aneuploid (18%, n=82) or Euploid (28%, n=120)

Fig 3



Euploid and Aneuploid embryos have similar:

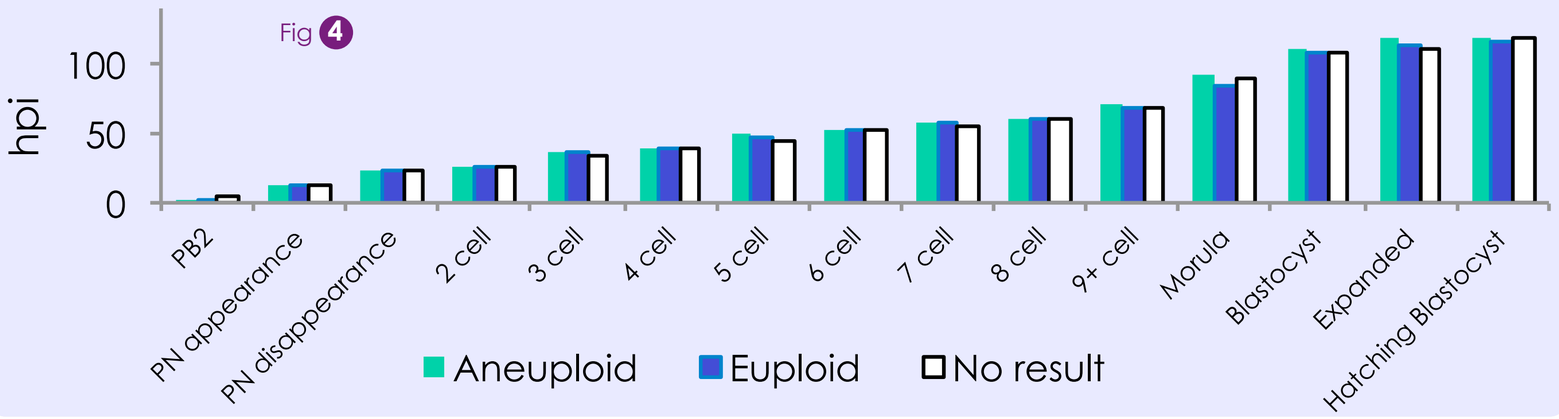
- Morphokinetic timings Fig 4



- Blastulation rate (43%, n=143 Aneuploid vs 40%, n=98 Euploid)
- Fragmentation at 2, 4 and 8 cell stages
- Cell evenness at 2, 4 and 8 cell stages
- Multinucleation
- Incidence of direct division from 1 to 3 cells and from 2 to 5 cells
- Trend for earlier compaction and blastulation was observed in Euploids compared to Aneuploids.

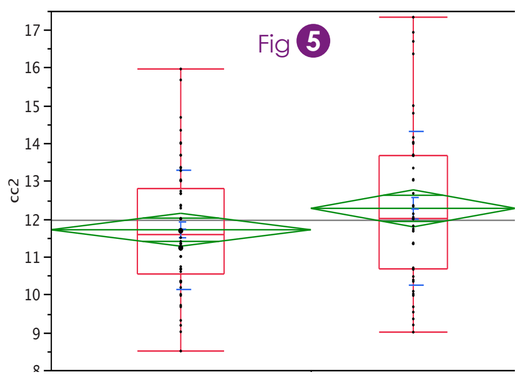
Common (fragmentation and multinucleation) and novel markers of embryo development (irregular division, 'direct' or rapid cleavage from 1 to 3 or 2 to 5 cells and very slow development t2>32hpi, t5>70hpi) appear to be linked to a higher frequency of aneuploidy although significance was not seen.

Fig 4



5. Second cell cycle (cc2) is faster for aneuploid embryos than for euploid embryos (p=0.047)

Fig 5



Stimulation protocol did not significantly affect the euploid to aneuploid ratio.

	ANEUPLOID	EUPLOID
Antagonist	13	9
Long Agonist	69	109
Short Agonist	14	11

Further Work

Differences between euploid and aneuploid embryos are being observed in the timing of later developmental events and will be scrutinised as numbers increase. Further work will involve more detailed assessment of the second cell cycle length and embryo ploidy.

The ploidy status of the embryo in this study was inferred from analysis of either the polar body or the trophectoderm. When sample numbers are increased, these will be looked at independently.

Conclusion

Euploid and aneuploid embryos have similar morphokinetics, degree of fragmentation, evenness and multi nucleation. While aberrant morphokinetic parameters may indicate a higher probability of aneuploidy, morphokinetics parameters alone are not sufficient to ensure selection of an euploid embryo for transfer. PGS remains a more definitive tool for euploid embryo selection but can be used in conjunction with morphokinetic evaluation.

With thanks to Niels Birger Ramsing for assistance with JMP data analysis

A morphokinetic selection model designed for early cleavage stage embryos using live birth as the successful measure of outcome.

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[3] *CARE Manchester , 108-112 Daisy Bank Road , Victoria park, Manchester, M145QH*
[4] *CARE London Park Lorne, 111 Park Road, London, NW8 7JL*
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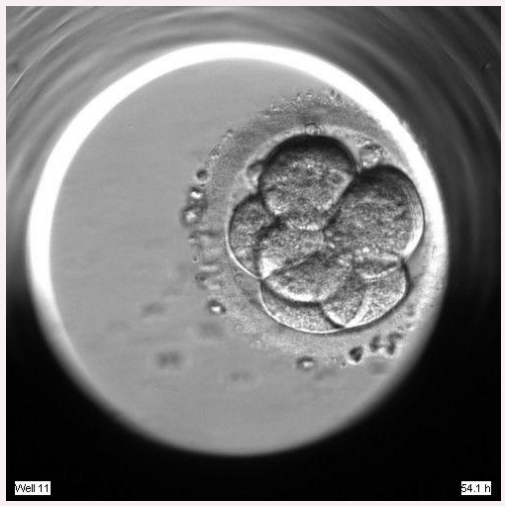
[6] *Intus Analytics, Denmark*



Introduction

A novel approach to creating a predictive algorithm based on live birth outcome derived from linear combinations of morphokinetic variables for 713 early cleavage embryos using principle component analysis (PCA).

Background

Timelapse monitoring of embryos and the use of defined morphokinetic markers to improve embryo selection is well documented, (Herrero et al 2013). Published evidence that a model based on such markers can distinguish between embryos with different implantation potential, and significantly improve ongoing pregnancy rate over standard incubation,(Rubio et al 2015).

This algorithm uses live birth outcome in preference to foetal heart as the outcome measure of success for early cleavage (day 3) embryos. The algorithm can be applied to both IVF and ICSI cycles by adopting the corrective value (delta IVF); the time taken for the sperm to travel to the oocyte and penetrate the oolema. Principle component analysis identifies variables within the data with a strong linear correlation that are predictive of live birth outcome.

Method

Morphokinetic variables and 713 known live birth (LB) outcomes, following day 3 transfer, (positive or negative) for IVF and ICSI embryos were annotated using the Embryoscope™ (Vitrolife, Sweden) between 2012 and 2014. The validation data set of foetal heart outcome (n=519), from 1.12.13 to 1.10.14 ,was used to validate the model built on the live birth training dataset.

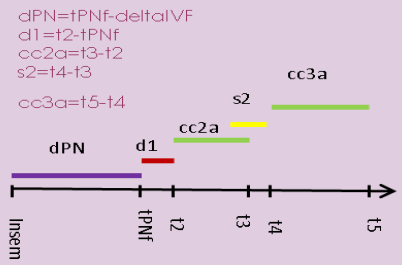
The principle component coefficients was derived from the training data set and recursive partitioning performed. The following morphokinetic variables were used in the creation of the principle components in line with published guidelines for time lapse nomenclature (Ciray et al 2014) : tPNF (time to PN Fading), cell division events of t2,t3,t4,t5, and dynamic events of dPN(tPNF-deltaIVF) where delta IVF equals 1.95hrs for IVF insemination and 0hrs for ICSI insemination, D1(t2-tPNf), CC2a(t3-t2), S2(t4-t3), CC3a(t5-t4) and cells D3(64) cell stage at 64hrs post insemination(hpi) see figure1.

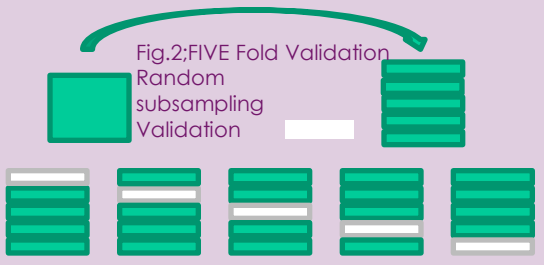
Principle components (PC) are linear combinations of these variables.

PC=a*dPN+b*d1+c*cc2a+d*s2…….. where a,b,c,d... are constant coeffiecients.

This PCA results in 5 principle components (PC1 to PC5) which under went recrusive partitioning to classify the PC's in a decision tree. To strenghten the classification model 5 fold validation (Figure 2) was performed, reducing the classification set to PC1,PC2 and PC4. The classification was validated against the foetal heart data not used in the model building (Figure 3). This produced the scaling values for the final predictive model.

Figure 1 :Timelapse Nomenclature





Outcome

Three classes were identified in the model (Figure 4) Class A =0.24 KID ratio, Class B=0.12 KID ratio and Class C= 0.067 KID ratio, with the area under the ROC curve (AUC) as 0.64 indicating good predictive value of the model (Figure 5).

Figure 4: Early Cleavage (Day 3) Model

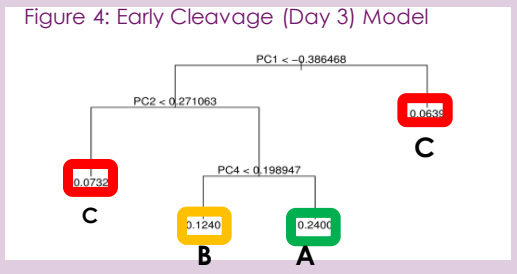
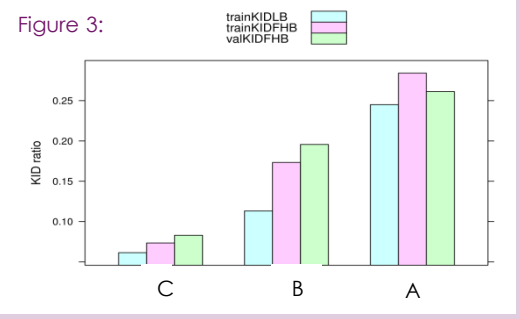


Figure 5	FHB n in bracket	LB n in bracket
KIDr Class A	0.293(92)	0.24(100)
KIDr Class B	0.164(110)	0.124(113)
KIDr Class C	0.086(486)	0.067 (518)
AUC	**0.641**	**0.641**

Figure 3:



Discussion

This model is derived from data from known live birth outcomes. The model uses a novel combinations of morphokinetic variables to generate principle components used in a predictive classification model. A model devised from live birth outcome may be seen as the ultimate measure for prediction of an embryo's success but the model could be limited by patient factors such as the uterine environment which may prevent a competent embryo achieving a live birth. This model is restricted to early cleavage stage embryos and may not be transferable to other clinics.

References:Ciray,N., Campbell,A., Agerholm,I., Aquilar,J., Chamayou,S., Esbert,M., Sayed,S., 2014. Proposed Guidelines on the nomenclature and annotation of dynamic human embryo monitoring by timelapse user group. Hreprd 0, pp.1-11

Herrero,J., Messeguer,M., 2013. Selection of high potential embryos using timelapse imaging: the era of morhokinetics. Fert and Steril 99, 4, 1030-1034.

Rubio,I., Galan,A., Larreategui,Z., Ayerdi,F., Bellver,J., Herrero,J., Meseguer,M.,2014. Clinical validation of embryo culture and selection by morphokinetic analysis: a randomised, controlled trial of the embryoscope.Fert and Steril 102,5,1287-1294

CAREfertility

Morphokinetic comparison of 1620 human embryos resulting in, or failing to reach, live birth.

Alison Campbell[1], Simon Fishel[1], Efstathios Theodorou[1], Sue Montgomery[1], Rachel Smith[1], Darren Griffin[2], Mette Laegdsmand[3]
[1] *CARE Fertility Group, Nottingham, UK*
[2] *University of Kent, Canterbury, UK*
[3] *Inctus Analytics, Arhus, Denmark*

Study question: **How do the timings and distributions of morphokinetic variables of human embryos resulting in, or failing to reach, live birth compare with each other and the entire unselected cohort of time lapse monitored embryos?**

Answer: **Significant differences exist in the distribution of specific morphokinetic variables of transferred embryos resulting in live birth, and those that did not; these differed depending on the stage of embryo transfer.**

What is known already?

Several time lapse derived morphokinetic variables have been proposed as indicators of embryo viability, and algorithms published to predict blastulation, aneuploidy risk or implantation potential. A randomized controlled trial has demonstrated the positive impact of morphokinetic based, compared with standard morphological, embryo selection. Morphokinetic information derived from transferred embryos where birth outcome is known, is limited, but may offer the most clinical value.

Study design

This is a retrospective, descriptive, multi-centre cohort study of morphokinetic variables from 15,485 embryos, in 3101 treatments including 1620 transferred embryos with known birth outcome cultured and time-lapse monitored following a standardised culture and annotation protocol, between 7/5/11 and 1/5/14. Data was compiled from six IVF clinics, following the same operating procedures for embryo culture, time lapse imaging and annotation using the EmbryoScope™ (Vitrolife, Sweden). Man Whitney U test was used to compare distributions.

Results

❖There is a wide distribution of morphokinetic parameters during the preimplantation development of human IVF embryos, even in controlled, standardised culture conditions.
❖For 1042 day 3 selected and transferred embryos, the distributions differed significantly between embryos failing (KIDneg), or succeeding (KIDLB), to reach live birth ($p<0.0001$) for the **duration of pronuclear appearance (dPN)**, calculated from second polar body extrusion to pronuclear fading. For this morphokinetic variable, the variance in KIDneg embryos was greater than for KIDLB. Interquartile ranges were 4.33 hours and 3.28 hours respectively. Figure 1. For 630 day 5 selected and transferred embryos, the distribution of dPN, did not differ significantly between the two groups.
❖**Duration of the second cell cycle** (time to reach three cells minus the time to reach two cells) was compared for 1692 embryos with known outcome. For day 3 and day 5 selected and transferred embryos, there was no significant difference between the bimodal distributions of KIDneg and KIDLB transferred embryos ($p=0.45$ and 0.74 respectively). Variance, in KIDneg was greater than in KIDLB embryos. Interquartile ranges were 4.08 hours and 1.66 hours for day 3 transferred embryos and 2.02 hours and 1.84 hours for day 5 transferred embryos (KIDneg;KIDLB respectively).
❖The time from pronuclear fading to **start of blastulation (relSB)** (Figure 2), and to the **full blastocyst stage (relB)** (Figure 3) were highly significantly different between embryos KIDneg and KIDLB embryos. ($p< 0.00001$ and <0.001 respectively). The variance in KIDneg was greater than in KIDLB embryos. Interquartile ranges were 10.12 hours and 7.17 hours respectively for relSB and 8.15 hours and 6.64 hours respectively.
❖No significant differences were found between the two embryo groups for the time from pronuclear fading to the time to reach **five cells (rel5)**, or the durations from the five cells to **start of compaction (dLS)** or **duration of blastulation (dB).**

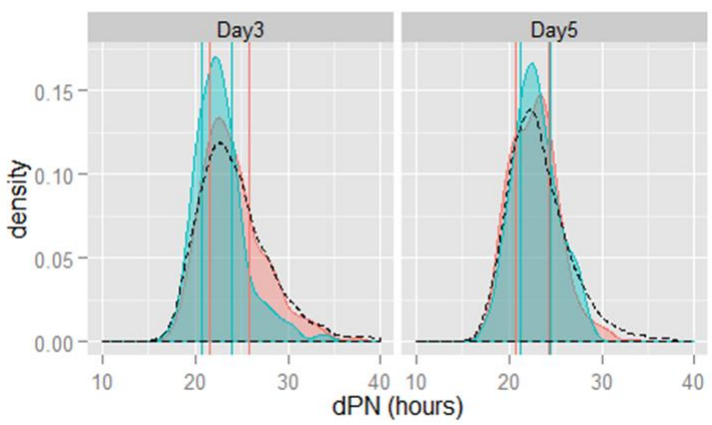


Figure 1, for duration of pronuclear stage (dPN), shows the distribution and interquartile range of KID negative (red), KID positive live birth (green) and all embryos created (dashed black line), by day of embryo transfer (day 3 or day 5). For day 5, the distributions were not significantly different.

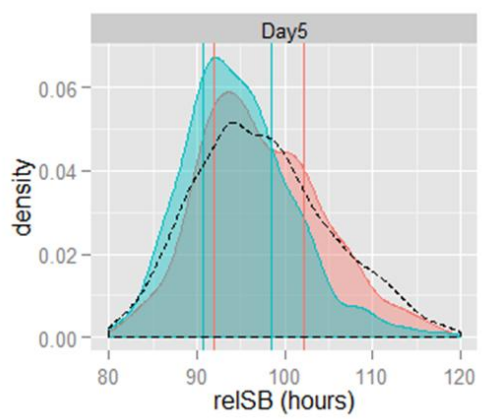


Figure 2, for time from pronucler fading to start of blastulation (relSB), shows the distribution and interquartile range of KID negative (red), to full KID positive live birth (green) and all embryos created (dashed black line).

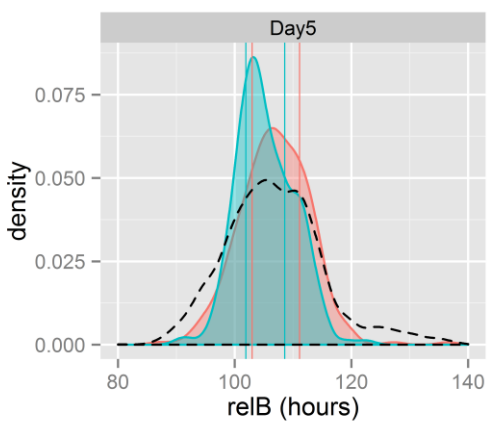


Figure 3, for time from pronuclear appearance blastocyst stage (relB) shows the distribution and interquartile rang of KID positive live birth (green) and all embryos created (dashed black line).

Conclusions

This study helps elucidate the broad distribution and variance of cleavage and developmental timings in human preimplantation embryos, and identifies morphokinetic candidates for human embryo selection in IVF treatment. Using healthy live birth as the outcome measure allows a more robust embryo selection and has potential to improve clinical outcome.

9.3 Appendix 3

Standard operating procedures for use of the EmbryoScope, annotation and application of algorithms for selection (CAREmaps) and quality assurance of annotation, devised and used at all CARE Fertility laboratories.

9.3.1 EmbryoScope Annotations and CAREmaps

Embryoscope™ Annotations and CAREMaps

Applies to:	Embryology
Written by:	Samantha Duffy/Louise Best/Alison Campbell
Issued by:	Alison Campbell
Approved by:	Executive Directors
Issue date:	17 September 2019
Review date:	16 September 2021
Reference:	Corp Lab 59b, issue 18

1.0 **EmbryoViewer Use**

All staff must have password access to the viewer to help track annotations. Designated staff should have administrator access to allow the user defined settings and models to be created or altered.

(For detailed use of the EmbryoViewer please refer to the EmbryoViewer Software v 4.1 User manual).

- The home screen on the EmbryoViewer corresponds to the EmbryoSlide arm, showing the location of each patient culture dish within the EmbryoScope.
- Select a patient record on the viewer screen then the "patient details" tab on the left to input patient information. Complete patient details for each new slide that is inserted into the EmbryoScope via the EmbryoViewer at the time of slide insertion. **This information is vital for future data analysis.**

(Whilst populating the fields in the patient details section duplicates the information we collect on CIS, a 'data for slides' agreement has been signed between CARE and Vitrolife Fertilitech so that they can use the unidentifiable patient data for evaluation).

- Once patient details have been completed, press "View running" tab to return to the home screen.

1.1 **Patient confidentiality**

- Identifying patient information must only be added to the 'Patient name' box on the EmbryoScope and EmbryoViewer. Comments boxes should not be used for identifying comments or details.
- The EmbryoViewer is automatically set to never export identifying patient information to Vitrolife. This can be seen by opening the "Settings" tab from the home screen and then clicking on the "FTP" tab.
- When the 'Team Viewer' icon is opened and used by Vitrolife Fertilitech to trouble shoot or upgrade the system, identifying patient data is not available for viewing and will not appear on the EmbryoViewer.
- A third party agreement is in place between Vitrolife and CARE Fertility.

2.0 **Annotation**

Each patient embryo must be annotated daily during its development.

- Once an embryo is selected on the viewer its development can be viewed by either clicking the play button, or by rolling to a particular time point using the "Griffin technology" hand tool.
- Highlight any developmental changes that occur in the oocyte/embryo using the annotation tools at the specific time point they took place.
- Should one embryologist question the detail/timing or type of annotation, of a developmental event made by a colleague, a second opinion should be requested before the annotation is altered.

The minutes/hours post fertilisation are shown in the bottom left hand side of the embryo images and is logged chronologically when an annotation is added either:-

1. Automatic - Using the automatic buttons (shown in blue column below).
2. Fixed term comments - Entering them in the comments field (shown in peach column below). Comments added via the comments box should always use the same terms, as shown in the peach column below to allow sorting of data.
3. User defined variables - In the EmbryoViewer software version 4.0 additional comments that are used regularly can be made into dropdown options to aid annotation speed and ensure consistency across CARE units. These can only be set by an administrator by going into "Settings" from the home screen and then clicking on the "Annotations" tab. Up to five "user defined variables" are available with 5 values possible for each variable. More values can be added but only the first five are visible on a data export.

Currently (June 2014) these are:-

	Parameter	Value
1	ICM	1
		2
		3
		Necrosis
2	Trophectoderm	1
		2
		3
		Necrosis
		Collapse
3	Early Obs.	Merged cells
		PB2/PB1 over 45^{o} apart
		4 Cell planar
		Single vacuoles (measure if large)

		Multiple Vacuoles (measure largest)
4	Compaction	Start compaction
		M1
		M2 (score % frags)
		Recompaction post decompaction
5	Approved study	Variables must be agreed by Director of Embryology

User defined variables are set by the CAREMaps focus group only and local variations cannot be introduced without prior approval from this group.

The table below is a summary of agreed annotations/ comments to ensure standardisation:-

Annotation summary table SEE SOP APPENDIX 2 for images

Observation to annotate on the embryo viewer	**Button present for automatic annotation**	**User-defined variable or Exact phrase for 'comment' box Then press return to log.**	**Comment/Detail**
1. Appearance of second polar body	PB2		Click when PB2 appears fully extruded with discrete membrane
1a. Non adjacent PB2/PB1 extrusion	User defined variable: Early obs	Early obs PB1/PB2 over 45° apart	Click at time of extrusion when PB2 is over 45° apart from PB1 see diagram appendix 4
2. Appearance of pronuclei	PN appeared		Click the automatic button to record as soon as the first PN is visible
3. Appearance of pronuclei Record only when clearly visible – the first frame that there is no doubt of the number of PNs.	0PN 1PN 2PN 3PN >3PN Even/uneven		Record as soon as clearly visible. Or 0pn at 18hours hpi. If degenerate, there is an option to record 'dead'. Scroll up past HB in the "Cells" automatic tab Record for 2pn only even or uneven based on the size of the PN's in relation to each other
4. Fading of pronuclei	PN faded		Click the automatic button to record as soon as PNs are no longer visible
5. Record when each cleavage is completed	2 cell – 9+ cell		Blastomeres appear to have a discrete membrane. Do not wait for cells to round up
5a Record evenness of blastomeres	Even/uneven **(2 cell, 4 cell and 8 cell only)**		Score at the time point of new cell number Score as even if the smallest and largest cells less than 20% different
5b Record fragmentation	0-10% 10-20% 20-50% 50%-100% **(2 cell, 4 cell 8 cell and M2 only)**		Score at the time point of new cell number at 2,4,8, cells and M2
5c Presence of multinucleation	Number of multinucleated cells visible		Total number of cells with more than one nucleus within it **Multinucleation is assumed to be absent if not annotated**
6. Compaction starts		Start compaction	Use user defined variable button to record when the very first signs of compaction are visible
7. Morula formation	Morula ('M')		

7a Grading		Compaction: M1 or M2 If M2 score the % fragments see 5a	User defined button record the appearance of the morula according to CARE's grading scheme- M1 no excluded material/ M2 excluded material present
8. Blastulation starts	tSb (time start blastulation)		Record when the very first signs of a cavity forming are visible. Rewind images to check
9. Full Blastocyst	B (Blastocyst)		Fluid filled cavity present. Last frame before it begins to increase the embryo diameter.i.e. as full as it can get without any size increase
9a Grading		ICM: 1,2 or 3 Trophectoderm: 1,2 or 3 Select 'Necrotic' where areas of necrosis seen for ICM and T	Using user defined variables Grade ICM and Trophectoderm as per Corp Lab 14. Can be entered in same time frame
10. Expanded blastocyst	EB (Expanded blastocyst)		
10a Grading		ICM: 1,2 or 3 Trophectoderm: 1,2 or 3 Select 'Necrotic' where areas of necrosis seen for ICM and T	Using user defined variables Grade ICM and T as per Corp Lab 14. Can be entered in same time frame
11. Hatching blastocyst	HB (Hatching blastocyst)		When herniation begins
11a Grading		ICM: 1,2 or 3 Trophectoderm: 1,2 or 3 Select 'Necrotic' where areas of necrosis seen for ICM and T	Using user defined variables Grade ICM and T as per Corp Lab 14. Can be entered in same time frame
12. Hatched blastocyst		Hatched	When (if) out of zona
13. Other observations; Merging cells, reduction in cell number due to cell fusion		Early obs: Merged cells	Use the user defined variable button to record merged cells. This is not compaction but 'reverse cleavage' When merged cells are observed • Click merged cells • Do not alter cell number • Next cell division do not annotate as no cell number increase – embryo has returned to previous annotated cell count.
4 cell Planar division		Early obs: 4 cell planar	Use user defined variable to highlight flat or one plane divisions from 2cell to 4cell. Normal division is tetrahedral where the plane of cleavage are at right angles to each other see pictures appendix 2, figure 5
Single Vacuoles		Early Obs: Single vacs (measure)	Annotate the presence of one vacuole at any stage of development, measure the size using the tool on the viewer page
Multiple Vacuoles		Early Obs: Multiple vacuoles (measure the largest)	Annotate the presence of vacuoles at any stage of development, measure the size of the largest vacuole using the tool on the viewer page.

Recompaction post decompaction		Compaction: recompaction post decompaction	When has been classed as M but has decompacted. Annotate once recompacted can rescore Morula if change in appearance see 7a grade
Expanded blast contracts, losing fluid cavity		Trophectoderm: Collapse	Use the user defined variable button to record full collapse of the blastocoel cavity in B or greater classified embryos. Record at end of collapse before rexpansion
Necrosis in the ICM or trophectoderm cells		ICM: necrosis Trophectoderm :necrosis	User defined variable button to record the presence of necrosis at each site
Smooth endoplasmic reticulum clusters		SERC	Enter as a comment when observed
Filopodia		Filopodia first appearance (write number in comments box if more than one) Filopodia - additional appear (write number in comments box) Filopodia - disappear (write number in comments box) Filopodia - all disappeared No filopodia observed	Use the user defined variable 5 button to record the presence/ absence of filopodia and their activity.
Ploidy		Euploid No result Not tested For aneuploidy results, give detail only e.g. +21, -14	Type the outcome of PGS for each oocyte/embryo on the bottom of patient details page under 'embryo description

Use icons * ? etc to mark the fate of each embryo	Colour coding denotes transferred, frozen, to be decided and discarded (green, blue, yellow/orange, and red respectively). User defined values in Early obs should be highlighted as amber for easy reference.
Selection of embryo(s) for ET	When the CAREMaps models has been used to overrule regular morphological selection this must be recorded (on the excel spreadsheet)
Peculiarity	When a rare and strange phenomenon or artifact is observed, in order to allow these to be retrieved in the future for study or presentation, in the comments box at the time of the event, enter **peculiarity -** ********** (give brief detail)

3.0 **Interruption - Pausing/ending a slide**

- If the slide needs to be removed from the EmbryoScope temporarily i.e. to remove bubbles affecting the image or to remove embryos for transfer, the slide is selected on the EmbryoScope touch screen followed by the Pause button.
- The slide will be moved to the correct position for removal and the door LED will show green.
- On returning the slide select re-insert.
- If a slide must be ended and reinserted (e.g. for a service), on returning to the EmbryoScope, the same treatment number cannot be used. The suffix 'int' must be used in these cases, as described below.

Slide exclusion category		Format of Tx no.
Interrupted side	-	12345int
Test side	-	12345test (or test)
Research	-	12345res

Test or research slides must be given a suffix in order to identify them (as above). Deletion of slides or data from the EmbryoViewer should be undertaken with extreme caution, with a witness and following authorisation. It may only be performed by the designated administrator or Laboratory Manager. It must always be justified and documented. The automatic data extraction under development will remove erroneous slides and data and as a result, negate the need for such deletion of information.

4.0 Embryo selection

Grade embryos cultured in the EmbryoScope, as per Corp lab 14, this should be used in conjunction with the CAREMaps models.

Click 'compare and select' and record the score for each cultured 2pn. The embryo/s with the highest score should be selected for transfer, whilst also considering morphology and any other 'peculiarities' seen during development.

For day 2 and day 3 transfers use the current EC (early cleavage) model in the compare and select function.

4.1 EC 4- Day 2
Cleavage stage The model was created using the following variables dPN, D1, cc2a, S2 and cellsD2

Uses principal component analysis of the training data resulted in new variables (PC1,PC2,PC3…)

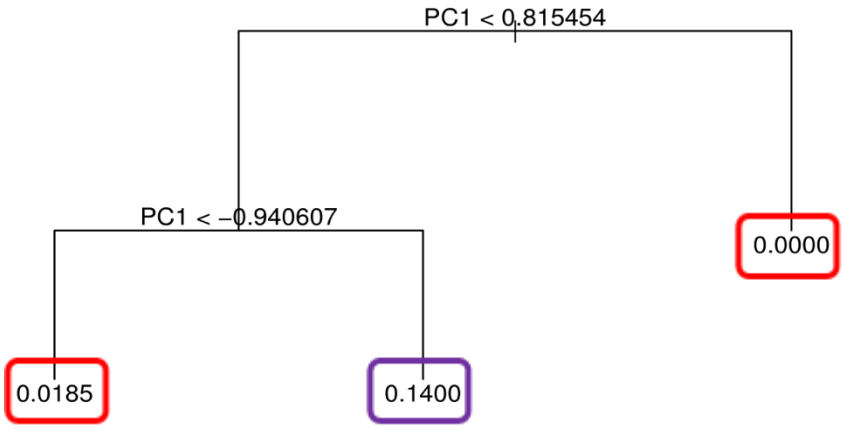
PC are linear combinations of the original variables

PC=a*dPN+b*d1+c*cc2a+……..

where a,b,c,d… are constant coefficients

		FHB n in bracket	LB n in bracket	Score
KIDr **(High)**	Class A	0.159 (176)	0.139 (193)	2
KIDr **(Low)**	Class C	0.045(133)	0.007 (142)	0
AUC		**0.643**	**0.712**	

4.2 **Additive model**

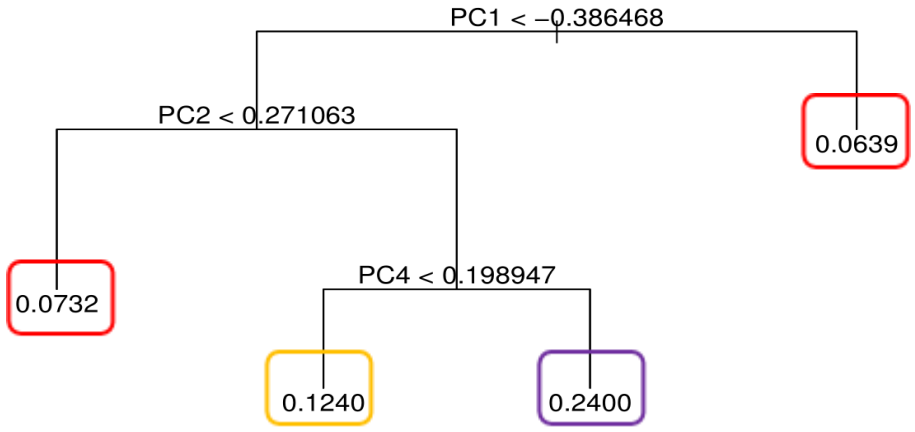


EC4 Day 3

The model was created using the following variables dPN, D1, cc2a, S2,cc3a and cellsD3

dPN=tPNf-deltaIVF

		FHB n in bracket	LB n in bracket	Scor
KIDr **(High)**	Class A	0.293(92)	0.24(100)	2
KIDr **(medium)**	Class B	0.164(110)	0.124(113)	1
KIDr Low	Class C	0.086(486)	0.067 (518)	0
AUC		**0.641**	**0.641**	



Custom Expressions for use with model Blast 4 (Feb 15)

Model uses insemination time as start time for the model so different models are required for ICSI and IVF

Expression Name	
relSBIVF	tSB-1.95
dB	tB-tSB

Score		Classification	Implantation potential
2	rtSB ≤ 93.1hrs	A	High
1	rtSB > 93.1hrs dB ≤ 12.5hrs	B	Medium
0	rtSB > 93.1hrs dB > 12.5 hrs	C	Low

Additive model

Blast 4 ICSI

var	Min	max	Weight
tSB	0	93.1	2
dB	0	12.5	1

Blast 4 IVF

var	Min	max	Weight
relSBIVF	0	93.1	2
dB	0	12.5	1

SEE APPENDIX 3 for the justification data for these models.

5.0 Embryo fate

At the end of the culture period slides are removed from the EmbryoScope by selecting the slide on the touch screen and press 'end'. The arm will move the slide to the correction position at the door for removal.

The colour of the embryo image on the viewer should reflect the embryo's fate:

- Green tick: transferred
- Blue snow flake: cryopreserved
- Red cross: discarded

- Amber question mark: undecided, i.e. irregular development pattern - any embryos that are coded as yellow during their culture period should be coded with the appropriate 'fate' colour at the end of their culture, i.e., red if discarded.

During EmbryoScope monitoring, 2PN embryos should be either grey or amber. Do not exclude them with red coding until disposal, as they may be required to ET.

6.0 Video uploading and sending CARE MAPS patients their videos via EmbryoScope™ Producer

The time lapse microscopy incorporated in the EmbryoScope culture system enables embryologists to upload videos of embryos at any time.

- Select an embryo using the EmbryoViewer
- Click on the "video" tab to the left of the embryo image
- Select the start and finish time of the video
- Click "generate"
- If the video is for patient use it should be saved in the "Patient videos" folder (under the patient video), currently found in the "Exported video" folder on the embryo viewer desktop.

Patients who have requested CARE MAPS/EmbryoScope culture have the opportunity to gain access to video footage of their transfer embryos either via an emailed web link or via the Patient Portal set up by CARE.

- Videos of the transferred embryo/s are created. If a patient has undergone a D.E.T. it is useful to name one embryo video (1) and the other (2) after the patient CARE no.
- The EmbryoScope producer software can be found on the EmbryoViewer desktop. Once opened, input the patient CARE no and press "Start".
- Clicking the "Select" tab will allow the relevant embryo video(s) to be chosen from the Patient Videos folder. Click "Go" and wait for the producer to generate a PIN.
- The Embryoscope producer software will alert the user to patient portal registration status once their CARE number is inputted and "Start" is pressed. (see relevant graphics in "Using the Embryoscope Video Producer Software" document no.15385)
- The Embryoscope producer will automatically link portal registered patients' CARE MAPS videos to their secure portal under the 'Videos' menu option at my.carefertility.com, in addition to uploading them to the original area at http://es.carefertility.com/). No PIN code needs to be sent to these patients via email unless specifically requested.

1 Patient feedback submitted from the automated questionnaire will be saved in spreadsheet format and can be accessed by CARE staff via the following web link: http://es.carefertility.com/dodaexport.php

7.0 Exporting data

The data produced by the EmbryoScope can be exported in excel spreadsheet, or csv, format at any point by following the instructions below:-

- Click the "View all slides" tab found on the EmbryoViewer home page.

- The patient slides appear in a list. Select the records required for export by highlighting the rows.
- Once the relevant slides have been selected (usually all, selected by pressing Ctrl and A), click "Export" tab.
- Follow the instructions to create an excel spreadsheet or csv file.
- Name the file with the export date and save in the "Exported data" folder found on the EmbryoViewer desktop

Associated documents:

- EmbryoViewer Software v 4.1 User manual revision 2012.12/EU
- Using the Embryoscope Video Producer Software, document no.15385

SOP Appendix 1 – images of annotation timings

Series of example time lapse images for morphokinetic variables tPN, t2, tSC, tSB and tB, from the same embryo.

Figure 1 – Pronuclear fading

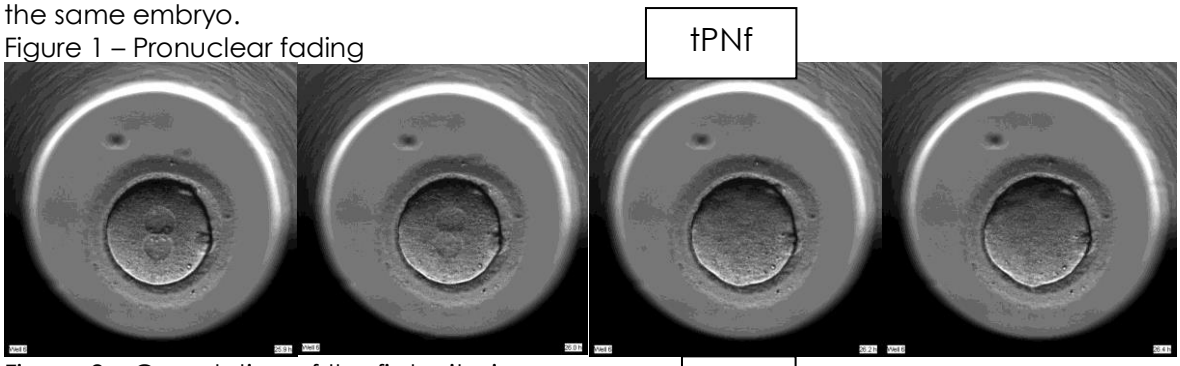


Figure 2 – Completion of the first mitosis

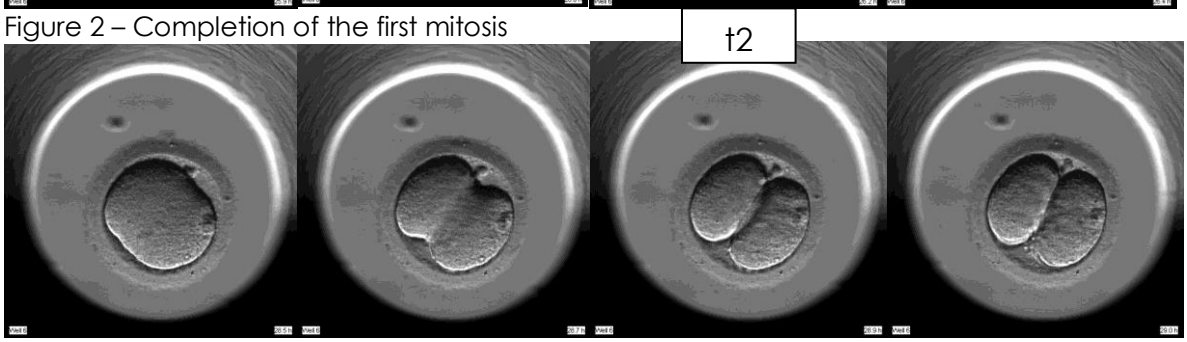


Figure 3 – Initiation of compaction

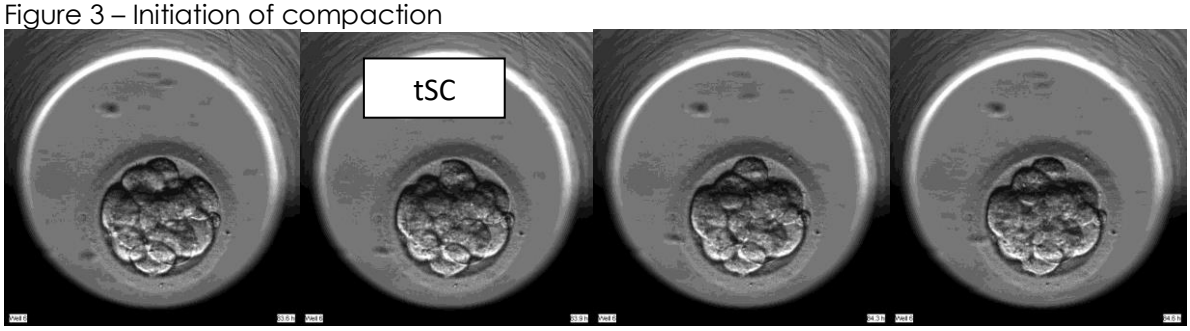


Figure 4 – Initiation of blastulation, and full blastocyst (images not sequential)

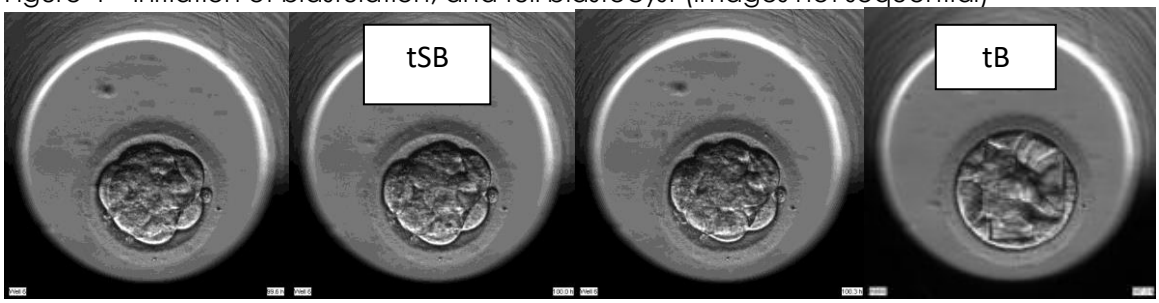
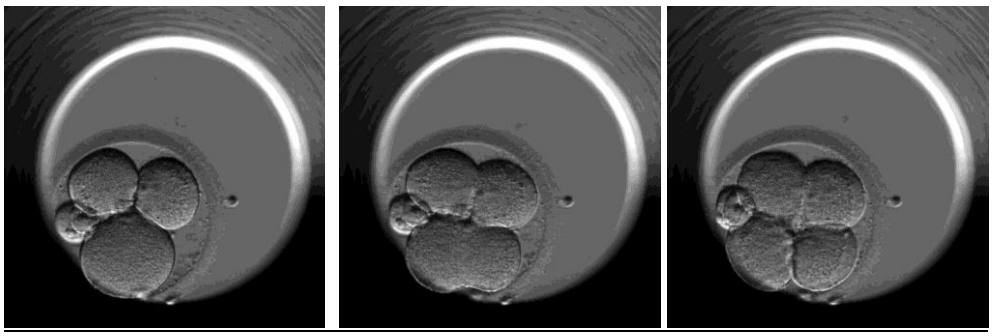


Figure 5 – Planar division at 4 cell stage



Video in:- CARE Media Library > Embryology > EmbryoScope Images for ATLAS > Planar 4 cell division

Link to play video:- 156497well 3 planar video.wmv

SOP Appendix 2 – Models on EmbryoScope

EC4- Day 2
EC4 Day3

Blastocyst selection model
Blast4 ICSI

Model Type
Additive

Creator
RACHELS

Custom Expressions

Name	Expression
dB	tB-tSB

Model Definition

Variable	Weight	Min	Max	Description	P(Variable)
tSB	2	0.0	93.1	Prefer	2, if 0.0 ≤ tSB ≤ 93.1 0, if 0.0 > tSB or tSB > 93.1
dB	1	0.0	12.5	Prefer	1, if 0.0 ≤ dB ≤ 12.5 0, if 0.0 > dB or dB > 12.5

Score = P(tSB) + P(dB)

Blast 4 IVF

Model Type

Additive

Creator

RACHELS

Custom Expressions

Name	Expression
relsBIVF	tsb-1.95
dB	tB-tSB

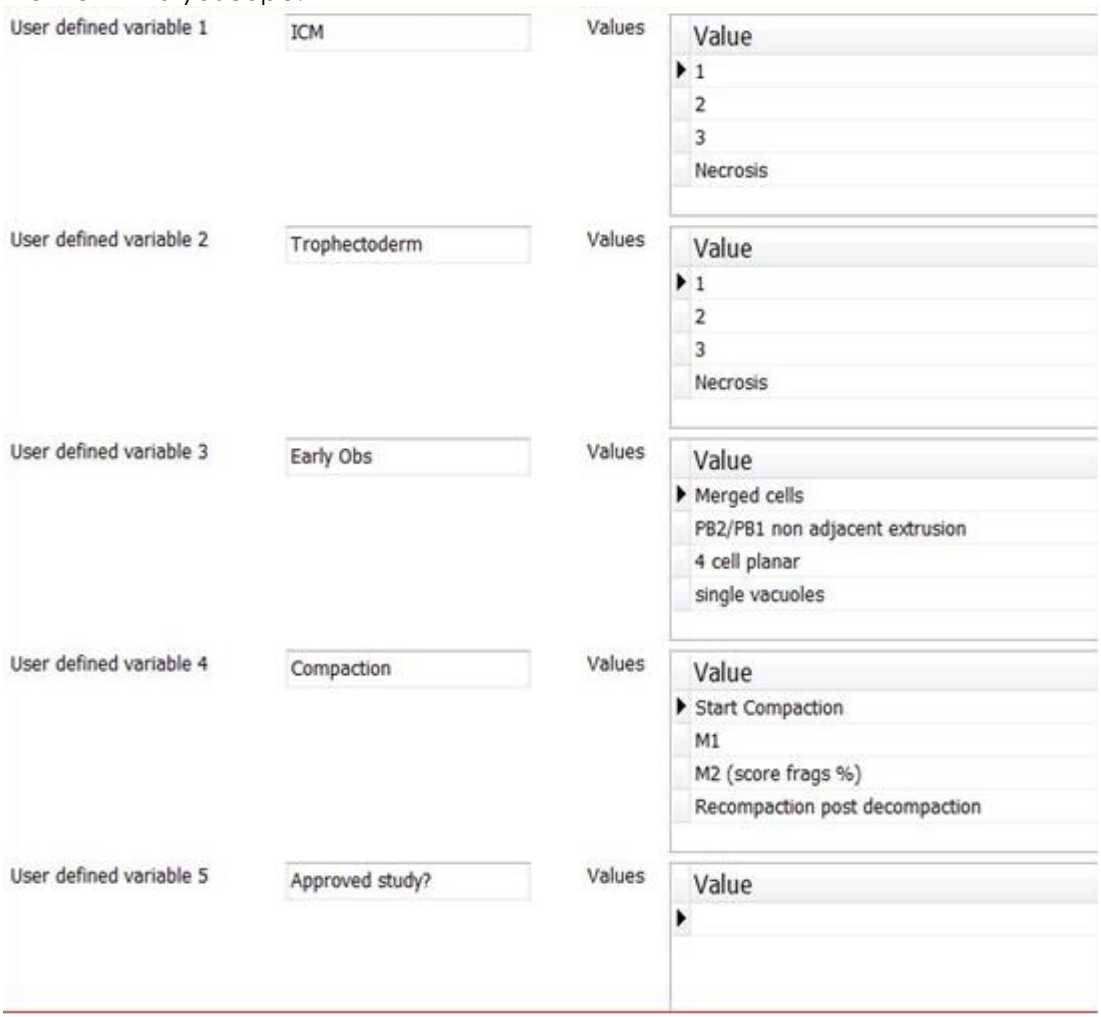
Model Definition

Variable	Weight	Min	Max	Description	P(Variable)
relsBIVF	2	0.0	93.1	Prefer	2, if 0.0 ≤ relsBIVF ≤ 93.1 0, if 0.0 > relsBIVF or relsBIVF > 93.1
dB	1	0.0	12.5	Prefer	1, if 0.0 ≤ dB ≤ 12.5 0, if 0.0 > dB or dB > 12.5

Score = P(relsBIVF) + P(dB)

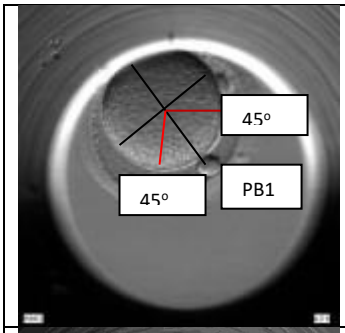
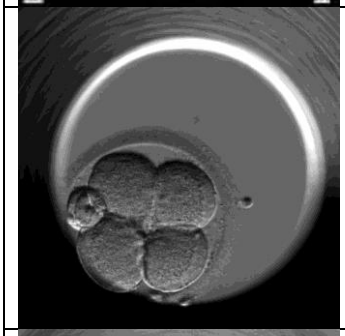
SOP Appendix 3 -User defined variables

Exert taken from Staff Tech notes – user defined variables– document manager
View on EmbryoScope:



Early Observations table- research evidence for deselection criteria

Parameter		Further information and training tips
Early obs. CARE Media Library > Embryology > EmbryoScope Images for ATLAS > 07-ACLK-Book Images>reverse cleavage: merged cells 151426	Merged cells (mark as amber)	Merged cells- abstract in appendix. Merged cells appear in lower model score. KID LB lower when seen (15.7 v 28.7%). De-select after model application if choice remains.

	PB2/PB1 non adjacent extrusion	Record at time of PB2 extrusion. ICSI study of PB2 at time of extrusion at CARE. n=198 KID embryos. CPR sig higher when PB2 extruded adjacent to PB1.
	4cell planar	Small (none CARE) study showed Tetrahedral 4 cell embryos have sig higher implantation rate (38 v 21%) than planer (flat/on one plane). n=98
	Single vacuoles (measure if large)	Poorly understood. ESHRE/Alpha consensus reported >14um can impact developmental potential. Time of presentation – late stage more detrimental Abstract BFS 2020 – extract . From 4462 transferred blastocysts, the incidence of single vacuoles was 2.6% (n=119) and multiple vacuoles 5.3%, (n=239). The live birth rate (LBR) for transferred blastocysts where multiple vacuoles were present was significantly lower than when no vacuoles were recorded, 20.9% vs 31.5% (p=0.01). Single vacuolated transferred blastocysts LBR was 28.5% vs 31.5% (NS). The appearance of both vacuole types in the late stage (>72hpi) resulted in significantly lower LBR compared to the rest of the cohort 12.3% vs 31.5% (p=0.01). Early appearance of vacuolation was not shown to be associated with reduced viability, LBRs were 27.1% vs 31.5%. see full abstract below.

EC4- Day 2
EC4 Day3

Blastocyst selection model
Blast4 ICSI

Model Type

Additive

Creator

RACHELS

Custom Expressions

Name	Expression
dB	tB-tSB

Model Definition

Variable	Weight	Min	Max	Description	P(Variable)
tSB	2	0.0	93.1	Prefer	2, if 0.0 ≤ tSB ≤ 93.1 0, if 0.0 > tSB or tSB > 93.1
dB	1	0.0	12.5	Prefer	1, if 0.0 ≤ dB ≤ 12.5 0, if 0.0 > dB or dB > 12.5

Score = P(tSB) + P(dB)

Blast 4 IVF

Model Type

Additive

Creator

RACHELS

Custom Expressions

Name	Expression
relsBIVF	tsb-1.95
dB	tB-tSB

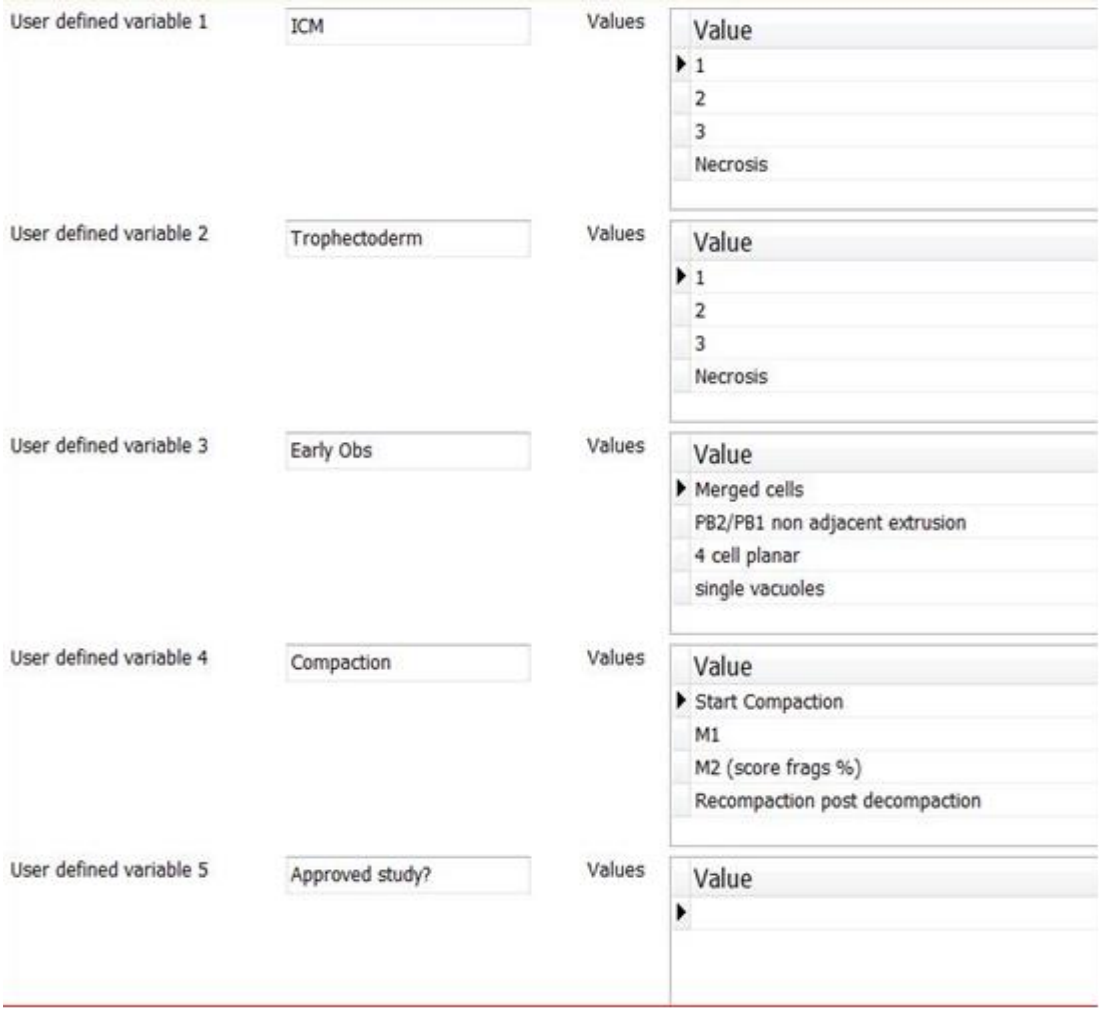
Model Definition

Variable	Weight	Min	Max	Description	P(Variable)
relsBIVF	2	0.0	93.1	Prefer	2, if 0.0 ≤ relsBIVF ≤ 93.1 0, if 0.0 > relsBIVF or relsBIVF > 93.1
dB	1	0.0	12.5	Prefer	1, if 0.0 ≤ dB ≤ 12.5 0, if 0.0 > dB or dB > 12.5

Score = P(relsBIVF) + P(dB)

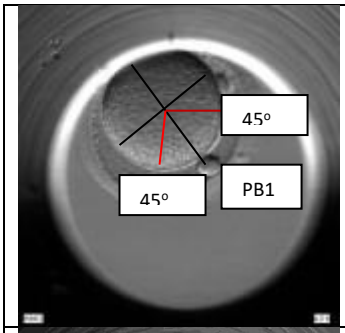
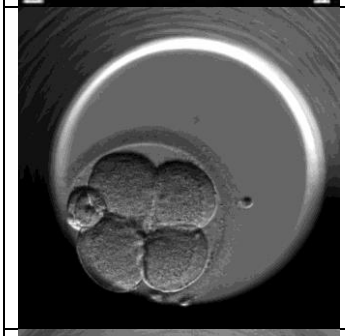
SOP Appendix 3 -User defined variables

Exert taken from Staff Tech notes – user defined variables– document manager
View on EmbryoScope:



Early Observations table- research evidence for deselection criteria

Parameter		Further information and training tips
Early obs. CARE Media Library > Embryology > EmbryoScope Images for ATLAS > 07-ACLK-Book Images>reverse cleavage: merged cells 151426	Merged cells (mark as amber)	Merged cells- abstract in appendix. Merged cells appear in lower model score. KID LB lower when seen (15.7 v 28.7%). De-select after model application if choice remains.

	PB2/PB1 non adjacent extrusion	Record at time of PB2 extrusion. ICSI study of PB2 at time of extrusion at CARE. n=198 KID embryos. CPR sig higher when PB2 extruded adjacent to PB1.
	4cell planar	Small (none CARE) study showed Tetrahedral 4 cell embryos have sig higher implantation rate (38 v 21%) than planer (flat/on one plane). n=98
	Single vacuoles (measure if large)	Poorly understood. ESHRE/Alpha consensus reported >14um can impact developmental potential. Time of presentation – late stage more detrimental Abstract BFS 2020 – extract . From 4462 transferred blastocysts, the incidence of single vacuoles was 2.6% (n=119) and multiple vacuoles 5.3%, (n=239). The live birth rate (LBR) for transferred blastocysts where multiple vacuoles were present was significantly lower than when no vacuoles were recorded, 20.9% vs 31.5% (p=0.01). Single vacuolated transferred blastocysts LBR was 28.5% vs 31.5% (NS). The appearance of both vacuole types in the late stage (>72hpi) resulted in significantly lower LBR compared to the rest of the cohort 12.3% vs 31.5% (p=0.01). Early appearance of vacuolation was not shown to be associated with reduced viability, LBRs were 27.1% vs 31.5%. see full abstract below.

SOP Appendix 4
Historic Modelling
2 EC3 (December 2013)

Custom Expressions for use with model EC3 (Dec 13)

Expression Name	
CC2	t3-t2
CC3	t5- t3
relCC2	(t3-t2)/(t5-t2)*100

Model weightings- Day2 ICSI (EC3 Dec 13)

MULTIPLICATIVE model

Score		Day3 ICSI EC3(Dec 13)	
	Expression	Min value	Max Value
0	CC2	0.0	2.0
0.25	T2	27.1	300.0
1	MN4	Info only	

Model weightings- Day3 ICSI (EC3 Dec 13)

MULTIPLICATIVE model

Score		Day3 ICSI EC3(Dec 13)	
	Expression	Min value	Max Value
0	CC2	0.0	2.0
0	CC3	0.0	5.0
0.25	T2	27.1	300.0
2	relCC2	44.0	47.0
1	MN4	Info only	

Model weightings- Day2 IVF (EC3 Jan 14)

MULTIPLICATIVE model

Score		Day3 ICSI EC3(Jan14)	
	Expression	Min value	Max Value
0	CC2	0.0	2.0
0.25	T2	29.1	300.0
1	MN4	Info only	

Model weightings- Day3 IVF (EC3 Jan 14)

MULTIPLICATIVE model

Score		Day3 ICSI EC3(Jan14)	
	Expression	Min value	Max Value
0	CC2	0.0	2.0
0	CC3	0.0	5.0
0.25	T2	29.1	300.0
2	relCC2	44.0	47.0
1	MN4	Info only	

Copy these models exactly as above (see appendix for photographs of model page on the EmbryoViewer).

Day 2 ICSI or IVF (+2.0h)

Score		Classification
0	Deselected if 0<= CC2 <=2.0h	Exclude *(where alternatives available)*
0.25	T2 >=27.1h	Low
1	T2 < 27.1h	Medium or High *If no 5 cell division*

If have 5 cell embryos on day 2 move to the day 3 model for improved selection

Day 3 ICSI or IVF (+2.0h)

Score		Classification
0	Deselected if 0<= CC2 <=2.0h Or 0<= CC3 <=5.0h	Exclude *(where alternatives available)*
0.25-0.5	T2 >=27.1h relCC2 between 0-100%	Low
1	relCC2 0-43.9% or 47.1%-100%	Medium
2	relCC2 44%-47%	High

9.3.1 CAREmaps quality assurance

CAREmaps Quality Assurance

Applies to:	Embryology
Written by:	Davina Hulme
Issued by:	Alison Campbell
Approved by:	Executive directors
Issue date:	28 March 2018
Review date:	28 March 2020
Reference:	Corp lab 59c

1.0 A CAREMaps quality assurance exercise should be completed by all embryologists within each unit quarterly to be presented by the lead embryologist at the CAREMaps focus group meetings.

2.0 Choose 3 embryos for all embryologists within your unit to annotate using the Corp Lab 61h Embryoscope annotation review sheet. Embryologists should annotate fully and write any user defined variable comments in the box provided so that timings as well as other observations can be monitored.

3.0 Collate the answers for your team into the template excel table saved in G: Embryology CARE: Embryoscope CAREMaps: quality assurance: QA tools: QA template excel sheet. There are 3 tabs to complete (timings, embryo descriptions & traffic light). This will allow direct comparison between embryologists and highlight any discrepancies that should be discussed at an internal QA meeting.

The first tab (timings) is set up to look at the annotated times for each embryologist. Enter your teams answers into the excel table which is automatically set up to show the mean, maximum and minimum ranges for all of the timings. Highlight any atypical answers to be discussed at your own internal CAREmaps QA meeting.

The second tab looks at the observations made whilst annotating. Please enter your teams' answers and highlight any discrepancies where some may have missed a particular user defined variable or embryo grading is different.

The third tab (traffic light) looks at how closely the answers recorded are to the mean i.e. + or – 10% and + or – 20% (ranges can be amended accordingly). This may highlight areas for discussion.

4.0 Calculate the intra class coefficient of variation (ICC) for the timings you wish to assess. This can be decided at each focus group meeting ready for the next QA exercise so that all timings are monitored regularly.

4.1 Open the ICC calculator located in G: Embryology CARE: Embryoscope CAREMaps: quality assurance: QA tools: ICC Calculator.

4.2 To calculate the ICC; e.g. for the 2 cell stage for the 3 embryos enter each embryologists (A-Z) annotated time for 2 cell for embryo 1 in the top row, embryo 2 in the next row and so on. The ICC value will be shown in the box to the right of the table, ICC (2, 4) is the average value. An ICC value of > 0.8 is considered strong agreement (Sundvall *et al.* 2013).

5.0 Upload the results into the CAREMaps focus group folder ready for discussion at the next global meeting.

6.0 An internal QA meeting should follow within each unit to discuss any discrepancies identified whilst observing the embryo videos as a group. This should ensure annotations are maintained in strong agreement.

Associated documents:

- Corp Lab 59a, issue 17 Embryoscope™ Culture
- Corp Lab 59b, issue 15 Embryoscope™ Annotations and CAREMaps
- Corp Lab 61h Embryoscope annotation review

References:

Sundvall, L *et al*. (2013) Inter - and Intra -observer variability of time lapse annotations. Human Reproduction vol28 no12 pp3215-3221.